

## SCREENING METHODS

The present invention relates to screening methods for drugs or lead compounds, enzymes, to polynucleotides encoding enzymes and to uses of enzymes and polynucleotides.

Protein kinase B (PKB) [1], also called RAC kinase [2] is the mammalian homologue of the viral oncogene product v-Akt [3] and has therefore also been termed c-Akt. The current interest in this enzyme stems from several observations. Firstly, it is activated within minutes in response to insulin or growth factors and activation is prevented by inhibitors of phosphatidylinositol (PtdIns) 3-kinase [4-6]. There is increasing evidence that PKB may mediate a number of the actions of insulin, including stimulation of glucose and amino acid uptake, glycogen and protein synthesis and cardiac muscle glycolysis (reviewed in [7, 8]) as well as regulation of the transcription of specific genes [9, 10]. Secondly, the PKB $\beta$  isoform is overexpressed in a significant percentage of ovarian and pancreatic cancers [11, 12] and the PKB $\alpha$  isoform in some breast cancers [2]. It appears that PKB provides a survival signal that protects cells from apoptosis induced in a variety of ways (reviewed in [8, 13]). The activation of PKB by gene amplification and other mechanisms may therefore contribute to the generation of malignancies that are able to flourish in the absence of extracellular survival signals.

PKB phosphorylates proteins and peptides at serine and threonine residues that lie in Arg-Xaa-Arg,-Xaa-Xaa-Ser/Thr- sequences [14]. In insulin signal transduction two physiological substrates of PKB appear to be the protein kinase - glycogen synthase kinase-3 (GSK3) [15, 16] and the cardiac isoform of phosphofructokinase-2 (PFK2) [8, 17]. Phosphorylation by PKB inhibits GSK3 activity leading to dephosphorylation and activation of glycogen synthase and

protein synthesis initiation factor eIF2B [18]. These events appear to contribute to the insulin-induced stimulation of glycogen synthesis and protein synthesis, respectively. PKB activates cardiac PFK2, which seems to underlie the insulin-induced stimulation of glycolysis in the heart. In the protection of cells against apoptosis, BAD appears to be one of the physiological substrates of PKB. This protein, in its dephosphorylated form, interacts with the Bcl family member Bcl<sub>XL</sub>, thereby inducing apoptosis in some cells. However, when PKB phosphorylates BAD at Ser136, it dissociates from Bcl<sub>XL</sub>, interacts with 14-3-3 proteins instead, and apoptosis is prevented [19].

The activation of PKB by insulin or growth factors requires its phosphorylation at two sites [20]. These are Thr308, which is located in the "activation loop" of the catalytic domain between subdomains VII and VIII, and Ser473 which is very close to the C-terminus in a hydrophobic Phe-Xaa-Xaa-Phe-Ser-Phe motif that is present in a number of protein kinases that play important roles in signal transduction [21]. The phosphorylation of both sites is prevented by inhibitors of PtdIns 3-kinase [20]. Thr308 is phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) [22, 23] and Ser473 by a protein kinase(s) having PDK2 activity. PDK1 may have PDK2 activity, for example when the kinase domain of PDK1 interacts with a region of Protein kinase C-Related Kinase-2 (PRK2) termed the PDK1 Interacting Fragment (PIF) or other polypeptide having a Phe-Xaa-Xaa-Phe motif. This converts PDK1 from a form that phosphorylates PKB at Thr308 to a form that phosphorylates PKB at both Thr308 and Ser473 (Balendran *et al* (1999) *Current Biology* 9, 393-404).

PtdIns 3-kinase converts phosphatidylinosotide 4,5 bisphosphate (PtdIns[4,5]P<sub>2</sub>) to PtdIns[3,4,5]P<sub>3</sub> which is then converted to PtdIns[3,4]P<sub>2</sub> by one or more 5'-phosphatases. PKB can only be activated by PDK1 *in vitro* in the presence of lipid vesicles containing PtdIns[3,4,5]P<sub>3</sub> or PtdIns[3,4]P<sub>2</sub> [22]. These 3-

- phosphoinositides bind to the pleckstrin homology (PH) domain at the N-terminus of PKB [24, 25], which is thought alters its conformation in such a way that Thr308 becomes accessible to PDK1. PDK1 also possesses a PH domain C-terminal to the catalytic domain [23], that interacts with PtdIns[3,4,5]P<sub>3</sub> and PtdIns[3,4]P<sub>2</sub> even more strongly than does the PH domain of PKB [26, 83]. The interaction of PDK1 with 3-phosphoinositides enhances the activation of PKB *in vitro*, probably by facilitating the interaction of PDK1 and PKB in lipid vesicles [27].
- 10 There is increasing evidence that PDK1 activates a number of protein kinases *in vivo*, such as p70 S6 kinase [28] and isoforms of PKC [29], by phosphorylating threonine residues that lie in positions equivalent to Thr308 of PKB. These protein kinases also contain the hydrophobic PDK2 consensus sequence that lies 160-165 residues C-terminal to the PDK1 phosphorylation site [8]. Thus other
- 15 substrates of PDK1 are also likely to be phosphorylated by a protein kinase with PDK2 activity.

- We have characterised yeast protein kinases that appear to be required for cell growth and that may have mammalian homologues that are able to rescue yeast cells (ie restore their ability to grow) that are not able to express the endogenous yeast protein kinase or kinases. Characterisation of these yeast protein kinases that may be functional homologues of mammalian protein kinases may allow improved methods of identifying compounds that may modulate the activity of signalling pathways in which the mammalian homologues are involved, or
- 25 compounds that may be useful as antifungal agents.

We have characterised non-mammalian proteins that may be functional homologues of PDK1 and human serum and glucocorticoid induced protein kinase (SGK), an enzyme whose catalytic domain is 70% similar to PKB.

### Screening methods using yeast cells

A first aspect of the invention is a method of identifying a compound which modulates the activity (preferably inhibits) to different extents of (a) a host yeast cell protein kinase or kinases and (b) a protein kinase derivable from a source other than the said host yeast cell that is equivalent to the said host yeast cell protein kinase or kinases, wherein a compound is exposed to

1) a first host yeast cell wherein the yeast cell is capable of expressing the said host yeast cell protein kinase or kinases and is not capable of expressing the said equivalent protein kinase

and

2) a second host yeast cell wherein the yeast cell is (a) not capable of expressing the said host yeast cell protein kinase or kinases and (b) is capable of expressing the said equivalent protein kinase derivable from a source other than the host yeast cell

and the effect of the compound on the phenotype of the said yeast cells is measured, wherein either

(1) the host yeast cell is a pathogenic yeast (preferably a yeast pathogenic to a mammal, preferably a human) and the source other than the host yeast cell is any source other than the host yeast cell

or

(2) the host yeast cell is any yeast and the source other than the host yeast cell is not a mammal.

It will be appreciated that the said first and second host yeast cells differ substantially only in the features indicated such that apart from these features the cells are essentially the same. This ensures that there is a reasonable expectation that the effect of the compound on the phenotype of the said yeast cells can be

attributed to the compound's effect on the modulation of the activity to different extents of the protein kinases as said. Thus, the first and second host yeast cells are from the same species and have substantially the same genetic content with the exception of the features indicated ie the capability to express the said host yeast cell protein kinase or kinases and the ability to express the said equivalent protein kinase derivable from a source other than the host yeast cell. It will be appreciated that the said first and second host yeast cells may differ in genetic content relating to the generation or selection of the said first or second host yeast cells (for example, in selectable marker genes used in recombinant techniques, as well known to those skilled in the art).

Typically, a compound that affects the viability of the first said yeast cell and the said second yeast cell differently, is identified. More particularly, the compound so identified is selected for further study.

15

It will be appreciated that the phrase "modulates the activity (preferably inhibits) to different extents" includes the meanings that (1) the host yeast cell protein kinase or kinases is/are modulated (inhibited or activated) and the protein kinase derivable from a source other than the said host yeast cell that is equivalent to the said host yeast cell protein kinase or kinases is not modulated (inhibited or activated), (2) the host yeast cell protein kinase or kinases is/are not modulated (inhibited or activated) and the protein kinase derivable from a source other than the said host yeast cell that is equivalent to the said host yeast cell protein kinase or kinases is modulated (inhibited or activated), and (3) that both types of protein kinase are modulated (inhibited or activated), but that one type is modulated (inhibited or activated) more than or differently to the other.

25

It will be appreciated that the modulation is judged by the effect on the phenotype of the said first and second yeast cells. It will be appreciated that the effect of the

compound on the protein kinase must be sufficient to cause a change in the phenotype being observed in order for any modulation to be detected. Preferably, the effect of a compound that modulates the said protein kinases on the phenotype of a cell is an alteration in the viability or ability of the cell to grow. Thus, it is preferred that the said host yeast cell protein kinase or kinases is desirable or essential for the viability or ability of the cell to grow.

By "protein kinase derivable from a source other than the said host yeast cell that is equivalent to the said host yeast cell protein kinase or kinases" (the "equivalent protein kinase") is included a protein kinase derivable from a source other than the said host yeast cell that is able to function in place of the said host yeast cell protein kinase or kinases in at least some aspect (ie in relation to at least one phenotypic characteristic), for example as judged by the viability or ability of a cell in which the host yeast cell protein kinase or kinase is replaced by the said equivalent protein kinase to grow. Thus by "equivalent protein kinase" is included a protein kinase that is able to function in place of the host yeast cell protein kinase or kinase in conferring on a host yeast cell a particular wild-type phenotype, for example the ability to grow under certain conditions.

For example, it is preferred that a yeast cell that is not able to express the said host yeast cell protein kinase or kinases is substantially not able to grow under given conditions, and that the said host yeast cell that is capable of expressing the said equivalent protein kinase is capable of growing under the same conditions. In the presence of a compound that inhibits the host yeast cell protein kinase or kinases but does not inhibit the equivalent protein kinase, the said first host yeast cell may not be substantially capable of growing but the said second host yeast cell may be capable of growing. Similarly, in the presence of a compound that inhibits the equivalent protein kinase but does not inhibit the host yeast cell

protein kinase or kinases, the said second host cell may be substantially incapable of growing.

Alternatively, it is preferred that a yeast cell that is able to express the said host yeast cell protein kinase or kinases expresses the said host yeast cell protein kinase at a level too low to permit growth of the cell at wild-type rates, preferably at a level at which the said cell is substantially not able to grow under given conditions, and that the said host yeast cell that is capable of expressing the said equivalent protein kinase expresses the said equivalent protein kinase at a level too low to permit growth of the cell at wild-type rates, preferably at a level at which the said cell is substantially unable to grow under the same conditions. In the presence of a compound that activates the host yeast cell protein kinase or kinases but does not activate the equivalent protein kinase, the said first host yeast cell may be more capable of growing but the said second host yeast cell may remain substantially incapable of growing. Similarly, in the presence of a compound that activates the equivalent protein kinase but does not activate the host yeast cell protein kinase or kinases, the said second host cell may be more capable of growing and the said first host yeast cell may remain substantially incapable of growing. Thus, for example, the host yeast cell protein kinase or kinases and the equivalent protein kinase may be expressed from a regulatable promoter and the cells may be grown under conditions in which the activity of the promoter is reduced. For example, the GAL1 promoter may be used, in a manner analogous to that described in Example 1. The GAL1 promoter is repressed in the presence of glucose, as known to those skilled in the art; thus, when cells are grown in the presence of glucose, expression from the GAL1 promoter will be reduced, in a manner analogous to that described in Example 1.

Thus, a yeast host cell which is incapable of expressing the said host yeast cell protein kinase or kinases may be substantially incapable of growing unless the

said yeast host cell is capable of expressing the said protein kinase derivable from a source other than the said host yeast cell that is equivalent to the said host yeast cell protein kinase or kinases at an sufficient level.

- 5 It will be appreciated that a compound that affects the phenotype, for example the viability or ability of the cell to grow, by a mechanism that does not directly involve the said protein kinase or kinases or equivalent protein kinase is unlikely to have a different effect on the first and second said host yeast cells. Thus, a compound that has a similar effect on both the first and second said host yeast
- 10 cells (for example, no effect on the viability of either type of cell, or, alternatively, toxic to both types of cell) may not be selected for further study, as the compound may not be affecting the activity of the said protein kinase or kinases or equivalent protein kinase. For example, the compound may be a non-specific cytotoxic agent affecting, for example, the integrity of the cellular
- 15 membrane.

It will be appreciated that a compound that is capable of modulating the activity of the said host yeast cell protein kinase or kinases and the said equivalent kinase may have a similar effect on both said first and second host yeast cells; however,

20 it will be appreciated that it may not be possible or convenient to attempt to distinguish such a compound from a compound that is affecting the phenotype by a mechanism that does not directly involve the said protein kinase or kinases or equivalent protein kinase.

- 25 It will be appreciated that the possibility of distinguishing compounds that may be acting directly on the said protein kinase or kinases or equivalent protein kinase from those that may be acting on other cellular components may be beneficial, for example as set out above. Thus, the method of the first aspect of the invention, which may allow such distinguishing, may be beneficial even if, for



example in the context of the intended use of any compound identified, it is not considered of great importance that the compound is not able to inhibit the said host yeast cell protein kinase or kinases, so long as it is able to inhibit the equivalent protein kinase (or *vice versa*).

5

Thus, for example, if it is intended to identify a compound that is capable of inhibiting a particular human protein kinase and that therefore may be useful in treating a human with a disease or condition that may be caused by elevated activity of the said protein kinase, it may not be essential for the medical usefulness of the compound that it does not inhibit an equivalent protein kinase in yeast.

10

Similarly, if it is intended to identify a compound that is capable of inhibiting a particular yeast (for example a pathogenic yeast) protein kinase and that therefore may be useful in treating a human with a disease or condition that may be caused by that pathogenic yeast (which compound may be identified by a screen according to the first aspect of the invention in which the host yeast cell is a non-pathogenic yeast cell, for example *S. cerevisiae* and the source from which the equivalent protein kinase is derivable is a yeast (for example *Candida*) which is capable of being a pathogen), it may not be essential for the compound to be medically useful that the compound does not inhibit an equivalent protein kinase in non pathogenic yeast, such as *S. cerevisiae*.

15

20

However, it will be appreciated that in other circumstances it may be desirable or essential for the medical or horticultural usefulness of the compound that the compound is (1) able to inhibit the said host yeast cell protein kinase or kinases, and (2) is not able to inhibit the equivalent protein kinase (or *vice versa*). For example, when the host yeast cell is a pathogenic yeast cell, a compound that

25

inhibits the host yeast cell protein kinase but not an equivalent protein kinase, such as a human protein kinase or a plant protein kinase, may be useful, for example as an anti-yeast (or anti-fungal) drug or plant protection product or in the design of an anti-yeast drug (or anti-fungal) drug or plant protection product.

5

A further aspect of the invention provides a method of identifying a compound which modulates the activity to different extents of (a) a protein kinase derivable from a first source and (b) a protein kinase derivable from a second source, both said protein kinases being equivalent to the same host yeast cell protein kinase or

10 kinases, wherein a compound is exposed to

1) a first host yeast cell wherein the yeast cell is (a) not capable of expressing the said yeast cell protein kinase or kinases and (b) is capable of expressing the said equivalent protein kinase derivable from the first source  
and

15 2) a second host yeast cell wherein the yeast cell is (a) not capable of expressing the said yeast cell protein kinase or kinases and (b) is capable of expressing the said equivalent protein kinase derivable from the second source  
and the effect of the compound on the viability of the said yeast cells is measured, and a compound that affects the viability of the first said yeast cell and  
20 the said second yeast cell differently, is identified. More particularly, the compound so identified is selected for further study.

The method of this aspect of the invention may allow a compound to be identified that may have an effect on cells of the said first source but that may not have an  
25 effect on cells of the said second source, or *vice versa*. Thus, it may be useful if the said first source is a mammal, for example a human, and the said second source is a parasite or pathogen (including an opportunistic pathogen), for example a yeast pathogen such as *Candida* spp. The terms parasite, pathogen and opportunistic pathogen are well known to those skilled in the art, and include

any source that may cause or contribute to a disease or condition of an organism, preferably a mammal, still more preferably a human.

It will be appreciated that the parasite or pathogen may be a parasite or pathogen of a plant; thus it may be useful if the said first source is a plant and the second source is a parasite or pathogen that may affect the said plant, for example is capable of causing rust. A compound that does not have an effect on cells of the said first source but that has an inhibitory effect on cells of the said second source may be useful in treating a plant that is affected by the said parasite or pathogen or in preventing the plant being affected by the said parasite or pathogen. Thus, it may be preferred that the first or second source are both not mammalian, or that either the first or second source is a pathogenic yeast cell.

It will be appreciated that the first and second source may both be plants; a compound that is capable of affecting cells of the first source and second source differently may be useful as a selective herbicide or a selective growth promoter. Thus, for example, the first source may be a monocotyledenous plant and the second source may be a dicotyledenous plant.

By "protein kinase derivable from" is included the meaning that the protein kinase is encoded by a nucleic acid, for example genomic DNA or mRNA, of the source. Thus, for example, a protein kinase derivable from a human may be a protein kinase encoded by a portion of a human genome or by a cDNA copied from human mRNA. It will be appreciated that the first and second source may be first and second tissue from an organism. In such a case, the nucleic acid may be the mRNA of a cell of the tissue. It is preferred that the protein kinase derivable from the first source is not identical to the protein kinase derivable from a second source. The said protein kinases may be tissue-specific or tissue-restricted isoforms, as well-known to those skilled in the art. Thus, the method

of the invention may be used to identify a compound that is able to modulate/inhibit a protein kinase derivable from a first tissue of an organism (ie the first source) to a different extent to an equivalent (non-identical) protein kinase derivable from a second tissue of the same organism (ie the second source).

Preferred embodiments of the invention include methods according to any of the above aspects of the invention wherein any of the appropriate said non-host yeast cell sources is a human (ie the protein kinase is derivable from a human).

10

It will be appreciated that if the protein kinase from the first and second sources are closely related then it may prove difficult to identify a compound that is able to inhibit the protein kinase from the first source but not the protein kinase from the second source, or *vice versa*. However, unless the said protein kinases are identical it may be possible to identify such a compound.

15

In these and other aspects of the invention described below, exemplary genera of yeast contemplated to be useful, either as a said host yeast cell or as a said source other than a said host yeast cell, in the practice of the present invention are

*Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryosaurus*, *Sporidiobolus*, *Endomycopsis* and the like.

20

By pathogenic yeast is included pathogenic yeast of any one of the genera *Candida* spp, *Blastomyces* spp, for example *B. dermatitidis*, *Coccidioides* spp, for example *C. immitis*, *Histoplasma* spp, for example *H. capsulatum*, *Sporothrix* spp, for example *S. schenckii*, *Aspergillus* spp, for example *A. fumigatus*, *A. flavus*, *A. niger*, *Phialophora compacta* (*Fonsecaea compacta*), *P. pedrosoi* (*F.*

25

*pedrosi*), *P. verrucosa*, *Cladosporium carrionii*, *Rhinocladiella aquaspersa*, *Cryptococcus* spp, for example *C. neoformans*, *Cephalosporium* spp, *Fusarium* spp, *Histoplasma* spp, for example *H. capsulatum*, *Pneumocystis carinii*, *Rhizopus* spp, *Rhizomucor* spp, *Madurella* spp, for example *M. mycetomatis*, *M. grisea*, *Pseudallescheria boydii*, *Paracoccidioides* spp, for example *P. brasiliensis*, *Prototheca* spp, for example *P. wickerhamii*, *Epidermophyton* spp, *Microsporum* spp, *Trichophyton* spp, and *Malassezia* spp, for example *M. furfur* (*Pityrosporum orbiculare*).

10 It will be appreciated that by "yeast" we include "fungi" and, in particular we include the pathogenic fungi of the genera *Aspergillus*, including *Aspergillus fumigatus*, *Cryptococcus*, including *Cryptococcus neoformans*, and *Histoplasma*, including *Histoplasma capsulatum*.

15 It will be appreciated that *Saccharomyces* spp, for example *S. cerevisiae* are not considered pathogenic yeasts. *Schizosaccharomyces* spp, for example *S. pombe* are not considered pathogenic yeasts.

It will be appreciated that, in a simple embodiment, the host yeast cell is rendered  
20 not capable of expressing the said host yeast cell protein kinase or kinases by virtue of a mutation or mutations in the gene or genes encoding the said host yeast cell protein kinase. Typically the mutation is any mutation that prevents expression of an active protein kinase; it is particularly preferred if the mutation is one which cannot be spontaneously reversed, for example a deletion of all or  
25 part of the gene encoding the protein kinase.

The host yeast cell which is capable of expressing the said equivalent protein kinase from a non host yeast cell source is most conveniently made by

introducing into a suitable yeast a genetic construct which encodes and is capable of expressing the said equivalent protein kinase.

It will be appreciated that the host yeast cell parental strain may not be wild-type. For example, particularly in relation to *S. cerevisiae* as the host yeast cell, mutant strains containing Ade<sup>-</sup> or Leu<sup>-</sup> or Ura<sup>-</sup> mutations may be used as the parental strain to allow selection of plasmid uptake.

It will be appreciated that a constitutive or inducible promoter may be used in the expression of the non-host yeast cell protein kinase. Examples of constitutive promoters well known in the art are the *adh* or the SV40 promoter. As discussed above and in Example 1, the GAL1 promoter may be used. Alternatively, the promoter from the gene encoding the host yeast cell protein kinase may be used to express the non-host yeast cell protein kinase. For example, the promoters from the Pkh1 or Pkh2 genes may be used, in a manner analogous to that described in Example 1.

It is preferred that the exogenous polynucleotide is stably maintained in the yeast. It is further preferred, though not essential, that the exogenous polynucleotide is stably integrated into the yeast genome.

Transformation of cell hosts is accomplished by well known methods that typically depend on the type of vector used and host cell. Transformation of *Saccharomyces* and related cells is described in Sherman *et al* (1986) *Methods in Yeast Genetics, a Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* 275, 104-109 may also be useful. *Schizosaccharomyces pombe* may be transformed following LiCl treatment or by electroporation.

Conveniently, a Bio-Rad Pulse Controller may be used for electroporation of *S. pombe* cells. The technique of electroporation of yeast is disclosed in Becker & Guarente (1990) *Meth Enzymol* 194, 182.

- 5 *S. cerevisiae* may be transformed by, for example, methods analogous to those described in Example 1.

It is particularly preferred if, in relation to the first aspect of the invention, the host yeast cell is incapable of expressing the protein kinase Pkh1 and/or Pkh2.

10

Thus, a further preferred embodiment of the invention is a method of the invention wherein the said host yeast cell protein kinase or kinases is Pkh1 and/or Pkh2, wherein Pkh1 is the polypeptide encoded by open reading frame YDR490c of *S. cerevisiae* or equivalent open reading frame in yeast other than *S. cerevisiae* and Pkh2 is the polypeptide encoded by open reading frame YOL100w of *S. cerevisiae* or equivalent open reading frame in yeast other than *S. cerevisiae*.

15

Pkh1 from *S. cerevisiae* may have the following amino acid sequence:

20 MGNRSLTEADHALLSKPLVPTSAEHTQTQEYPRPFVDGSNSQSGSELQAS  
PQGQFGEKALTSTNRFIPLANDDPGMQHEMGLDPSMRRRREEWAERGA  
IVKDVVDPATGELTKHVVKMGIKDFKFGEQLGDGSYSSVVLATARDGKK  
YAVKVLKEYLIRQKKVKYVTVEKLALQKLNGTKGIFKLFFTFQDEASLY  
FLLEYAPHGDFLGLIKKYGSLNETCARYYASQIIDAVDSLHNIGIIHRDI  
25 KPENILLDKNMKVKLTDFTAKILPEEPSNTADGKPYFDLYAKSKSFVGT  
AEYVSPPELLNDNYTDSRCDIWAFCILYQMLAGKPPFKAANEYLTFOKVM  
KIQYAF TAGFPQIVKDLVKKLLVRDPNDRLTIKQIKAH LFFHEVNFEDGS  
VWDDNPPEIQPYKINAEAMKPLQKVSESDTTVKMANLQLAGNGHADTPLQ  
APAATSQEH SVISMTAATAAFNKDYTSQPKLGSKSSTSVRSASNNTDREV

IQKKVSKNRASVSSPSISTTSRGKDNRSRSSDAFWSRYLQNMDERVLLMK  
EVALSTRNLEDSPVGLENVALDYKNPLDIEPPTDSAGKFYKKMFLITNLG  
RALVFVKRRSLSMWEEQEFELQFELELNDVEKIRFISDQVLEIDGSRTIF  
IGCKERAVLMKLWKLIHNGMTAKPKVVSPKSDHKMFDFILQKRQNTKKK  
5 NQAPPVPQSNRLINGLPDRCILKTPEEGALHTKRPTSLQTRSSSNYSKLL  
ARSTQMRKNMTRTDEK

(766 aa complete sequence)

*Pkh1* from *S. cerevisiae* may be encoded by the following nucleotide sequence:

10 ATGGGAAATAGGTCTTTGACAGAGGCAGACCACGCCCTGCTGTCCAAGCC  
CTTGGTACCGACATCTGCGGAACATACACAAACGCAAGAGTATCCTCGTC  
CTTTCGTAGATGGCAGCAATTCTCAGAGCGGGTCTGAACTACAGGCTTCT  
CCACAAGGTCAGTTTGGAGAAAAGGCATTGACTAGTACTAATCGCTTCAT  
TCCCCTGGCAAATGATGACCCGGGTATGCAGCACGAGATGGGTCTTGATC  
15 CCTCAATGAGGCGTAGAAGAGAAGAATGGGCAGAACGTGGTGCGGCAAAA  
ATCGTCAAAGATGTTGTCGACCCAGCTACGGGGGAGTTAACTAAGCATGT  
TGTC AAGATGGGAATAAAGGACTTCAAGTTTGGAGAGCAACTCGGGGATG  
GATCATATTCTAGTGTTGTTCTGGCTACCGCCCGTGATTCGGGCAAGAAA  
TATGCAGTAAAAGTGTTGAGTAAAGAATATCTGATCCGTCAAAAAAAGT  
20 TAAATACGTCACAGTGGAGAAATTGGCTTTGCAGAAGCTGAATGGCACCA  
AGGGCATATTCAAGCTTTTCTTCACTTTCCAGGACGAGGCAAGCTTGTAT  
TTCCTTCTAGAATATGCCCCCCACGGTGATTTCTTGGGCTTGATTAAGAA  
ATATGGATCTTTAAACGAGACATGTGCACGCTATTATGCGTCGCAGATCA  
TCGATGCCGTTGACTCCTTGCATAATATCGGCATTATTCACAGGGATATC  
25 AAGCCCGAAAACATATTGCTCGACAAAAATATGAAAGTGAAGTTGACGGA  
TTTTGGTACAGCCAAAATTTTACCGGAGGAACCTTCGAACACCGCAGATG  
GCAAGCCTTATTTGATTTGTATGCTAAGTCGAAATCATTTGTTGGTACC  
GCAGAATATGTTTCTCCTGAGCTACTGAATGATAATTATACAGATTCCCG  
TTGTGACATTTGGGCATTTGGTTGCATATTGTACCAAATGCTTGCGGGAA  
30 AACCGCCTTTTAAAGCTGCCAATGAATATTTGACATTCCAAAAAGTAATG



AAGATTCAATATGCGTTTACTGCAGGTTTTCCGCAAATAGTAAAAGATTT  
AGTTAAAAAACTATTAGTTAGGGATCCAAATGATAGATTGACCATAAAGC  
AGATCAAAGCACACCTCTTTTTCCATGAAGTCAACTTTGAAGATGGTTCT  
GTTTGGGATGATAATCCACCGGAGATACAGCCATATAAAATAAATGCAGA  
5 GGCGATGAAGCCCCTGCAAAAGGTTTCTGAATCTGATACCACTGTCAAAA  
TGGCCAACCTTCAGCTGGCTGGTAATGGACATGCAGATACTCCCCTGCAA  
GCACCAGCAGCGACCTCTCAAGAGCATTCTGTGATCAGTATGACTGCAGC  
AACCGCCGCATTCAATAAAGATTATACAAGTCAACCCAAATTGGGGAGCA  
AGTCAAGCACATCTGTTAGATCTGCCTCCAACAACACAGATCGCGAGGTA  
10 ATTCAAAAGAAGGTTTCAAAAAATCGCGCATCTGTATCATCTCCTTCAAT  
TTCTACTACATCACGGGGGAAAGATAATAGAAGTCGCTCTTCTGACGCCT  
TCTGGTCACGCTACTTGCAAAATATGGATGAACGTGTCTTGTTGATGAAG  
GAGGTAGCGCTTTCACACGAACTTAGAGGACTCACCTGTAGGTCTTGA  
GAACGTGGCTCTGGACTACAAGAACCCTCTTGATATCGAGCCTCCTACTG  
15 ATAGTGCAGGCAAATTTTACAAGAAAATGTTTCTAATAACAAACCTAGGC  
AGAGCACTTGTTTTTGTCAAGAGAAGAAGTCTCAGCATGTGGGAAGAACA  
GGAATTTGAATTGCAATTCGAACTAGAGTTGAATGACGTTGAGAAGATAC  
GCTTTATAAGTGATCAAGTCCTTGAAATTGACGGTTCCAGGACGATTTTC  
ATAGGATGCAAAGAGAGAGCAGTTTTAATGAACTATGGAATTAATACA  
20 TAATGGAATGACCGCCAAACCTAAAGTAGTATCGCCGAAGTCGGACCATA  
AAATGTTTGATAAGTTCATTCTTCAAAAAAGACAGAATACAAAAAAAAG  
AATCAAGCTCCTCCCGTACCTCAATCGAATAGGCTAATAAATGGTTTACC  
GGACCGTTGCATCTTAAAGACACCCGAAGAAGGCGCACTTCACACAAAAC  
GTCCCACTTCGTTGCAGACCCGATCGTCATCTAATTACTCAAATTGCTG  
25 GCAAGATCGACACAAATGCGGAAAAACATGACACGGACAGATGAAAAATG  
A

(Genbank accession number 927745; 2301 bp complete sequence).

*Pkh2* from *S. cerevisiae* may have the amino acid sequence:

30 MYFDKDNSMSRPLLPSDEQKLNINLLTKKEKFSHLDPHYDAKATPQRST

SNRNVGDLLEKRTAKPMIQKALTNTDNFIEMYHNQQRKNLDDDDTIKEVM  
INDENGKTVASTNDGRYDNDYDNNNDINDQKTLDNIAAGSPHMEKNRNKVKI  
EHDSSSQKPIAKESSKAQKNIIKKGIKDFKFGSVIGDGAYSTVMLATSID  
TKKRYAAKVLNKEYLIRQKKVKYVSIIEKTALQKLNNSPSVVRLFSTFQDE  
5 SSLYFLLEYAPNGDFLSLMKKYGSLETCARYYAAQIIDAI DYLHSNGII  
HRDIKPENILLD GEMKIKLTDFGTAKLLNPTNNSVSKPEYDLSTRSKSFV  
GTAEYVSPPELLNDSFTDYRCDIWAFGCILFQMIAGKPPFKATNEYLT FQK  
VMKVQYAFTPGFPLIIRDLVKKILVKNLDRRLTISQIKEHHFFKDLNFKD  
GSVWSKTPPEIKPYKINAKSMQAMPSGSDRKL VKKSVNTLGKSHLVTQRS  
10 ASSPSVEETHSTLYNNNTHASTESEISIKKRPTDERTAQILENARKGIN  
NRKNQPGKRTPSGAASAALAASAALT KKTMQSYPTSSSKSSRSSSPATTS  
RPGTYKRTSSTESKPFASKPPLSASVLSSKVPMPPYTPPMSPPMTPYDTY  
QMTPPYT TKQQDYS DTAIAAPKPCISKQNVKNSTD SPLMNKQDIQWSFY L  
KNINEHVLRTEKLDFVTTNYDILEKKMLKLNGSLLDPQLFGKPRHTFLSQ  
15 VARSGGEVTGFRNDPTMTAYSKTEDTYYSKNIIDLQLLEDDYRIEGGDL S  
ELLTNRS GEGYKCNQNSSPMKDDDKSESNNGSSVFSGKIKKLFHPTSAA  
ETLSSSDEKTKYYKRTIVMTSFG RFLVFAKRRQPNPVTNLKYELEYDINL  
RQQGT KIKELIIPLEMGTNHIVVIQTPYKS FLLSTD KKTTSKLFTVLKKI  
LNSNTNKIEKELLQRNQKVIERRTSSSGRAIPKDLPTSKSPSPKPRTHSQ  
20 SPSISKHNSFSESINSAKSNRSSRIFETFINAKEQNSKKHAAPVPLTSKL  
VNGLPKRQVTVGLGLNTGTNFKNSSAKSKRS

(1081 aa complete sequence).

Pkh2 from *S. cerevisiae* may be encoded by the nucleotide sequence:

25 ATGTATTTTGATAAGGATAATTCCATGAGCCCTAGGCCGTTATTGCCAAG  
TGATGAGCAGAAGCTAAACATTAATCTTCTAACGAAAAAGGAGAAATTCT  
CGCATTTAGACCCCCATTATGACGCAAAAGCCACTCCACAAAGAAGCACT  
TCGAATAGAAACGTTGGCGATTTACTTTTGGAAAAAAGAACCGCTAAGCC  
TATGATTCAAAGGCCTTGACGAATACGGATAATTTCAATTGAAATGTACC  
30 ATAATCAGCAGAGAAAAAATCTTGATGATGACACTATTAAAGAAGTAATG

ATTAATGATGAAAACGGAAAACTGTGCTAGTACCAACGACGGCAGATA  
TGACAACGATTACGATAATAACGATATTAATGACCAAAAACTTTGGATA  
ATATAGCGGGAAGTCCCCACATGGAAAAAATCGAAACAAAGTAAAGATT  
GAACATGACTCTTCATCTCAAAAACCAATAGCTAAAGAGTCATCCAAAGC  
5 CCAAAAAAATATAATCAAAAAGGGAATCAAGGACTTTAAATTTGGTAGTG  
TAATAGGTGATGGCGCGTATTCTACTGTAATGTTAGCGACGTCGATTGAT  
ACCAAAAAGAGGTACGCCGCAAAAGTACTAAACAAAGAATATTTAATACG  
CCAGAAGAAAGTCAAATACGTCAGCATAGAAAAACCGCCCTTCAAAGC  
TCAATAATTCTCCTAGTGTTGTGCGATTATTTTCCACTTTTCAGGATGAA  
10 TCAAGCCTATACTTTCTCTTAGAGTATGCCCCCAATGGGGACTTTCTTTC  
TTTAATGAAAAAATACGGTTCATTAGACGAAACCTGCGCACGATATTATG  
CTGCGCAAATAATAGATGCCATAGACTACTTACATTCCAACGGTATTATT  
CATAGAGATATAAAACCAGAAAATATTCTTTTAGATGGAGAAATGAAGAT  
CAAACCTGACTGATTTTGGTACTGCGAAGTTACTGAATCCTACAAATAATA  
15 GCGTTTCGAAACCAGAATACGATTTATCAACAAGGTCGAAATCTTTCGTT  
GGAACCTGCAGAATACGTATCTCCAGAACTTTTAAATGACAGTTTTACAGA  
CTATCGTTGCGATATTTGGGCCTTCGGATGTATACTTTTCCAGATGATTG  
CCGGAACACCACCATTCAAAGCTACCAATGAATACTTGACTTTCCAAAAG  
GTAATGAAAGTTCAGTACGCCTTTACACCAGGTTTCCCACTTATTATCAG  
20 AGATTTGGTTAAGAAAATCTTAGTAAAAAACTTAGACCGAAGATTGACGA  
TAAGCCAAATTAAGGAACATCATTTTTTCAAAGATTTGAATTTTAAAGAC  
GGCTCTGTTTGGTCAAAAACGCCTCCAGAGATCAAACCATATAAAATCAA  
CGCCAAATCCATGCAGGCAATGCCAAGCGGAAGCGATAGAAAACCTGGTGA  
AGAAATCAGTCAACACACTTGGCAAATCGCATCTAGTGAATCAAAGGTCA  
25 GCTTCAAGTCCCTCTGTTGAGGAACTACTCATTCAACCCTATACAATAA  
CAATACTCACGCTTCTACTGAAAGTGAAATATCAATAAAGAAGAGACCCA  
CTGATGAAAGAACAGCGCAGATACTTGAAAATGCAAGAAAGGGTATAAAC  
AATAGGAAAAATCAACCAGGCAAGAGAACACCAAGTGGTGCAGCTTCTGC  
TGCCCTAGCAGCTTCTGCTGCTTTAACCAAGAAAACCATGCAAAGCTATC  
30 CAACTTCTAGTTCGAAAAGTAGCAGGTCAAGCTCTCCTGCGACAACATCA

AGACCAGGAACTTATAAGCGTACTTCTTCTACAGAAAGTAAACCATTTCG  
CAAATCTCCACCTTTGTCAGCATCAGTTTTATCGTCAAAAGTCCCAATGC  
CTCCATACACACCTCCAATGTCGCCCCCTATGACACCATATGATACATAT  
CAAATGACACCTCCCTATACGACAAAACAGCAGGATTATTCTGATACCGC  
5 AATTGCCGCACCTAAGCCTTGTATTAGTAAGCAAAATGTTAAAAATAGCA  
CAGATTCTCCCTTGATGAACAAGCAAGATATTCAATGGTCCTTTTACCTG  
AAAAACATCAACGAACATGTACTAAGGACGGAAAAACTGGATTTTGTAC  
CACAAATTACGATATCTTAGAGAAGAAAATGCTTAACTAAATGGTTCAT  
TGTTAGATCCTCAACTGTTTGGTAAGCCTAGACATACTTTTTTATCCCAA  
10 GTAGCTAGGAGTGGGGGAGAGGTTACAGGTTTTCGAAATGATCCAACAT  
GACTGCTTATTCCAAAACAGAAGATACGTACTATTCGAAAAATATTATCG  
ATTTGCAGCTCTTGGAAGATGATTATCGAATTGAAGGAGGTGACTTATCG  
GAGTTGCTTACTAACAGAAGCGGAGAAGGGTACAAATGCAATCAAAACAG  
CTACCAATGAAAGACGATGATAAATCCGAATCTAACAATAAAGGAAGCT  
15 CTGTTTTTTCTGGCAAGATTAAAAAATTATTTACCCCTACCTCAGCAGCT  
GAAACGCTCTCTCCTCTGATGAAAAAACCAAGTACTATAAACGAACCAT  
TGTAATGACATCATTTGGAAGGTTTCTAGTATTTGCCAAGAGGAGGCAGC  
CAAATCCAGTTACAAATTTAAAGTATGAACTAGAATATGACATAAATTTG  
CGTCAACAGGGTACCAAATAAAAGAACTAATCATTCCCTTGGAATGGG  
20 AACTAATCATATAGTTGTGATTCAGACACCTTACAAGTCATTTCTTTTGA  
GCACTGATAAAAAAACCACGAGCAAATTGTTTACTGTTCTCAAAAAAATT  
CTTAATTCGAATACAAATAAAATAGAGAAAGAACTGTTGCAAAGAAACCA  
AAAGGTAATTGAAAGAAGAACATCATCATCCGGAAGAGCCATACCTAAAG  
ATCTTCCAACCTCCAAGTCTCCTTCGCCAAAACCCAGGACGCATAGCCAA  
25 TCTCCATCAATTTCAAAGCACAATTCGTTTTCTGAATCGATTAATAGCGC  
TAAGAGCAACAGATCAAGCAGAATTTTTGAAACCTTTATCAATGCCAAGG  
AACAAAATTCAAAAAAACACGCTGCTCCAGTACCGTTAACCAGTAAATTA  
GTTAACGGATTGCCAAAAAGACAAGTTACCGTGGGATTAGGTCTAAACAC  
AGGAACAAATTTCAAAAACCTCATCTGCAAAATCGAAGAGGTCGTAAT  
30 (Genbank accession no 1419952; 3247 bp complete sequence).

Equivalent open reading frames in yeast other than *S. cerevisiae* may be identified as such by methods well known to those skilled in the art and as described below.

5

The KSG1 gene (PDK1-like) from *Schizosaccharomyces pombe* may encode a polypeptide which comprises the following amino acid sequence:

MRNTHNPNETEASEDAENDTQSESDLSFDHGSSEKLNRLASLPKTQNSAIPQSNALN  
TTPNESTSQIDSSPKIPSAVPHISTPNPSSGASTPNIKRVSDFKFGGEILGEGSYST  
10 VLTATENSTKREYAIKVLDKRHIIKEKKEKYVNIEKEALCILSKHPGFIKLFYTFQ  
DAHNLYFVLSLARNGELLDYINKLGRFNEICAQYYAALIVDSIDYMHGRGVIHRDL  
KPENILLDDNMRTKITDFGSAKILNSSHGSHEEDTHHADKPQAHRSRFSVGTARYVS  
PEVLSDKIAGTASDIWAFGCILFQMLAGKPPFVAGNEYLTQFSILHLSYEIPPDIS  
DVASDLIKKLLVLDPKDRLTVDEIHQHPFFNGIKFDNTLWELPPRLKPFGHTSVL  
15 SLSVPNASNKHENGDLTSPLGVPSMVSASTNAAPSPVGTFNRGTLPCQSNLEEN  
KEWSSILQDDEKISKIGTLNVYSMSGINGNDAFRFFSSLFRKRKPRTFILTNFGRY  
LCVASDGEGRKTVKEEIPKISVGMRCRMVKNNEHGWVETPTKSWSFEDPNGPASA  
WVELLDKASSISLPFGNHSVTSFSRSIARSAV  
( > gi|3341488|gnl|PID|e1312259 protein kinase. *S. pombe*).

20

The KSG1 gene from *S. pombe* may comprise the following nucleotide sequence:

GGTATCTTCAGTTAAATCTTGCGTTGGTTGTCCTTTAATGAAGATTTGTTTCGTAAG  
CAAATATATGAATTCGCTAGCAAGATATTGCCATTCATACCAACCCTTTTCTTTA  
TGTTATATCTCCAAAATGAATTGTTTACAACACTAAATTGAAACAAATCAAACACA  
25 CTTTGTTCCTATTAGGTGTAGTGGAATGCAATTCTAAACAATGGCACTTTATTT  
TTGATTCTGTGCTGAACAAATAATTCATATTTATAAACTGAATGGTGCATAATTTT  
CTCTCTCTGTAAAATTGATACGGTAAGTTGAAATTCAAGTAAAATATGTTTCCCGC  
ATATTCAGCTACGATTCTGGTATAAAAGCTTCTAATGCTCTTCGTATATAAACTGA  
GTTATTCATTAAACCTAAGATCTTTTAAGTTATTGAAATTAAATCATATTGAATT  
30 CCACACCCGAAGGTAATGCTATTCAACCAGCCAAGACGAAAATACTTGGTTTGACA

ATTTTTTTACTTTTATGTAATGATTTTCTCTACTCCATTAAGAATTTGCGTATGAC  
TGTGTATACTCAGCGCTGATTCACATAGTAATATAAATCATTACCTTGATGAACTA  
CTTCTCTAGGCTCCTTAAAAAATAAATAAACTTATAGTTACTAAAAAAGCATGA  
TCTAAATCAAATGAATGAATATTTAGTTTATCTAAAATGTCGAATTCACTATTAAT  
5 TTTTCGCACTCACAACGATGCAAATCATAACCCGGATAGCAAAATCTTATATAACAA  
TAGTACAATAGTTGTATACTTGCAGTTATAAGCACTAATTTTACCGAATATGTGCA  
ATTTTCTTTTGATGACATTGCCGTCAATTCGTAGTTTACTGACACTGATTCACCTA  
CCCAGTACTCTCTTCACACCAGATATTGGTTATGCGAAATACGCACAATCCGAATG  
AAACTGAAGCATCAGAAGATGCAGAAAATGATACTCAAAGCGAATCCGACCTTAGT  
10 TTTGATCATGGATCAAGTGAAAACTAAACCGAGCTTCATTACCAAAGACGCAAAA  
TAGTGCCATCCCGCAGTCTAATGCTTTAAATACTACACCAAATGAGTCAACGTCTC  
AGATCGATTCATCGCCGAAGATTCCCTCTGCAGTCCCACATATCTCTACTCCAAAT  
CCGTCTAGCGGTGCATCGACTCCTAACATAAAACGAGTTTCGGATTTTAAATTTGG  
CGAAATCCTTGGAAGAAGGATCATACAGTACTGTATTAACAGCTACCGAAAACCTCAA  
15 CAAAAAGAGAATATGCTATTAAGGTGTTGGATAAAAGACATATTATCAAGGAAAAA  
AAGGAAAAATACGTCAACATTGAAAAAGAAGCTTTGTGCATCCTCTCTAAGCATCC  
TGGATTTATTAAGCTATTTTATACTTTCCAAGATGCTCACAATTTGTATTTTGTTT  
TGAGTCTTGCTCGAAATGGTGAAGTGTGGATTATATCAATAAGGTATGTATAAAT  
AATGTTTCATGCAGCTTGATGATGGATAGCTTACTGATTATTACAATCTTGTCTTTT  
20 TTCTTATTAAATGTTTTATTTTTTGCTTACACATAATTTAGTTGGGACGATTTAAC  
GAAATATGTGCACAATATTATGCTGCACTTATCGTTGACTCAATAGACTATATGCA  
TGGACGAGGAGTGATTCACCGTGACTTGAAGCCCGAAAATATTTTGCTGGATGATA  
ATATGCGTACAAAAATTACCGACTTTGGTTCAGCAAAAATATTAACTCTTCACAT  
GGCAGTCATGAAGAAGACACACACCATGCTGACAAACCACAGGCTCATTCTCGATC  
25 GTTTGTAGGAAGTCTCGTTATGTATCTCCTGAAGTTTTAAG  
TGATAAAATTGCTGGTACGGCTTCCGATATTTGGGCGTTTGGTTGCATTCTTTTCC  
AAATGCTAGCTGGCAAACCCCTTTTGTAGCAGGAAATGAATATTTGACATTCCAA  
AGTATTCTTCATTTGAGTTATGAGATACCACCTGACATATCAGATGTCGCTTCTGA  
TTTGATTAAAAAACTGCTAGTTCTAGATCCTAAAGATCGCTTAACTGTTGATGAAA  
30 TACACCAGCATCCGTTTTTTAATGGTATCAAATTTGATAATACCTTTGGGAACTT

CCTCCTCCCCGTCTTAAACCTTTTGGTCATACTAGCGTCCTCAGCCTTTCCGTTCC  
TAATGCATCTAACAACACGAAAATGGTGATTTGACCTCGCCTCTTGGTGTTCAT  
CGATGGTTTCAGCATCCACCAATGCTGCACCCTCTCCGGTTGGTACTTTTAACCGA  
GGCACTCTATTGCCGTGTCAATCCAACCTTGAGGAGGAAAACAAGGAATGGTCGAG  
5 TATTCTTCAAGACGATGAAAAAATCTCAAAAATTGGAACCCTCAATGTTTATAGTA  
TGTCTGGTATCAATGGAAATGATGCCTTTCGCTTTTTCTCCAGTCTTTTTCGAAAA  
AGGAAACCTCGGACCTTTATACTTACAAATTTTGGTCGATACCTGTGTGTTGCCTC  
GGACGGTGAAGGGCGAAAAACAGTTAAAGAAGAAATACCTATTAAGAGTGTAGGCA  
TGCGTTGTCGGATGGTAAAAACAATGAACATGGCTGGGTAGTTGAGACTCCAACA  
10 AAATCCTGGTCGTTTGAAGACCCGAATGGACCGGCTTCTGCTTGGGTGAGCTTCT  
AGATAAGGCTAGCTCTATTTCTCTTCCATTCGGTAATCATCTGTTACCAGCTTTT  
CAAGAAGCATTGCTAGAAGTGCTGTCTAATCTATCCTTTATGTCTATGAACACTTC  
CATCCATCCCCTCACTTTTTATTCTATCACTCTTTTTTCTGTTCTGTTCTGCATTA  
ATTTTCTTACGACCTTATTGATGTTTCAAGAAATTCTTTTTTAAGTTTTTCTCTTAC  
15 GTTCGTTGTTTAATTCGAAAAAAATCTTTATATCCGTGATCCTTCAGTGTGATTA  
CAAAATTAGATTGCCGTATGATTTCTGTATTTTGTATTAAACGGTCAATTGATTTT  
ATTTGCTTATTCTTATTATGGTCTATTTATTTTATATTTTTTACCTTAGCCTTAAA  
ATGTTAAAATGGTAAAAGTTATCGATCCAAGAACATTTCAACGTTTACTAGTTAAT  
ATACCTCTACCTACGTAGTAAAAAAATTTAAAGAAATTATTCCCAAAAGCCAGTTG  
20 TACATTCTCTCAATTTATGATTAATCTACAGATTAAGTTAATTATGCAAGTATGAC  
TTGTACATAAAAATTAGGTTTTCGCTTAATATCAGAAAAAAAACCTCAAGCATCAA  
TTTACTAGCATGATAAATAATGAATTACTTCTCCAAGTATGTTTTTGCGACACGAT  
C

(>gi|3341487|emb|X99280|SPKSG1 *S.pombe* KSG1 gene (PDK1-like))

25

A second PDK1-like gene (SPBC4C3.11) of *S. pombe* may encode a polypeptide which includes the following amino acid sequence:

MDLEHKRISRSTLPDYADPDYFEARGERNPVKQPSSNVVPGTSHIGSIKSPADYVF  
GDIIGDGSFSKVRRTDKKSWKEYAIKVLDKKYIVKENKVKYVNIERDSMMRLNGF  
30 PGISRLFHTFQDDLKLYYVLELAPNGELLQYIKKYRFLDENCVRFYAAEILSSIEY

MHSCGIIHRDLKPENILFDGNMHVKITDFGTAKILPPKYVNSPDYTTFPSSFVGT  
 EYVAPELLSRQVVSKSSDLWAFACVVYQMIVGSPPFHGSNPNNIFKKIMSLEYELP  
 KLLPPDIVPLFSLHFRIQPSDRSTTQQIKQFFPFATITWDNLWTQDPPPMQSF  
 RPNY  
 NIAIPNAPAYYRSNVTAAAAANAAAAFASASIVKHQETARRQELPTVNRFTAPTA  
 5 HYGYASLRSHQMPVDRLYYKLVPSSESI

(>gi|2832892|gnl|PID|e1249768 hypothetical protein kinase phosphorylase).

The second PDK1-like gene (SPBC4C3.11) of *S. pombe* may include the following nucleotide sequence:

10	acaacttcat	ttttcatgaa	aacctctttt	ataaatgatg	gatctggagc
	ataaacgcat	tagccgaagt	acattgccgg	attatgcgga	tcccgattac
	ttcgaggcta	gaggtgaaag	aaatccggta	aaacctcagt	cttccaacgt
	agtaccagga	acaagtcata	taggatcgat	caaatctccg	gcggattacg
	tttttggtga	cattatagga	gatggatcat	tctcaaagggt	aagttgatac
15	attgcttctc	agttcagaag	tttttcaacc	gcaggtcgat	taacctatgg
	accttcgatt	actgacgaat	aagttgtcta	atattcgtta	ggtgagaaga
	gcaactgata	aaaagagttg	gaaggagtac	gctatcaaag	tccttgataa
	aaaatatatt	gtcaaggaaa	ataagggttaa	gtatgtgaat	atagagagag
	attctatgat	gagacttaat	gggtttcctg	gtatctctcg	tcttttccat
20	acatttcagg	atgatttaaa	actttattat	gtgcttgaac	ttgcacccaa
	tggtgaactt	ttgcaataca	tcaaaaagggt	atattttttc	attagtctat
	tcattttttcc	tttattaact	aagctttggt	agtatcgttt	tcttgatgag
	aattgtgtgc	gcttttatgc	tgctgagatt	ttatcaagta	tcgagtatat
	gcactcctgc	ggtataattc	acagagatct	caagccagaa	aagtatgttt
25	gagtagtggt	cattaaatgt	tcgtttcctt	ttcctaattc	taacctattt
	tttagcattt	tatttgatgg	aaatatgcat	gtaaaaatta	ccgatttcgg
	cacagccaaa	atcctacccc	ctaaatatgt	aaatagccct	gattacacta
	cctttccaag	ctcctttggt	ggcactgcgg	aatatgttgc	tcctgaacta
	ttgtctagac	aagttgtttc	aaaatcgtaa	gaaaacctat	tatcccagtc
30	tatttttttt	ctgacaaata	tttaagttcc	gatttatggg	cttttgctg
	tgttgtttat	caaagtattg	ttggttcccc	tccttttcat	ggcagcaatc
	ctaataatat	tttcaaaaag	ataatgagcc	tggaatatga	gcttccaaag
	ctcttaccac	ctgatatcgt	tcctttgttt	agccatcttt	tccgtattca
	gccatctgat	cgatctacaa	cccaacaaat	aaaacaattt	cctttttttg
35	ctactattac	ttgggacaat	ttatggactc	aagatcctcc	tcctatgcag
	tcattccggc	ctaattataa	catagccatt	cctaagtctc	ctgcttatta
	tcgctcaaat	gtgacagccg	cagctgctgc	taatgctgcc	gcggcatttg



cttctgcattc cattgtaaag catcaggaaa ctgctcgacg tcaggagctt  
cctacggtaa atcgtttcac tgcaccaact gtcattatg gctatgcttc  
acttcgaagc catcagatgc ctggtgacag actttattac aagttggttc  
catcgtctga gtcgatac

5

The terms Pkh1 and Pkh2 may represent PKB-activating Kinase Homologues 1 and 2, respectively. Yeast, for example *S. cerevisiae*, cells that are capable of expressing either Pkh1 or Pkh2 or both are capable of growing, but yeast, for example *S. cerevisiae*, cells that are not capable of expressing either Pkh1 or  
10 Pkh2 are not capable of growing, as described in Example 1.

A protein kinase derivable from a source other than the said host yeast cell that is equivalent to the said host yeast cell protein kinase or kinases, for example Pkh1 and/or Pkh2, may be PDK1, for example mammalian, preferably human PDK1.

15 As described in Example 1, yeast, for example *S. cerevisiae*, cells that are not capable of expressing either Pkh1 or Pkh2 may be capable of growing if they are capable of expressing human PDK1.

The PDK1 may be plant PDK1. An example of plant PDK1 may be a  
20 polypeptide which comprises the following amino acid sequence:

MLAMEKEFDSKLVLQGNSSNGANVSRSKSFSFKAPQENFTSHDFEFGKIY  
GVGSYSKVVRAKKKETGTVYALKIMDKKFITKENKTAYVKLERIVLDQLE  
HPGI IKLYFTFQDTSSLYMALESCEGGELFDQITRKGRlseDEARFYTAE  
25 VVDALEYIHSMGLIHRDIKPENLLLTSdGHIKIADFGSVKPMQDSQITVL  
PNAASDDKACTFVGTAAYVPPEVLNSSPATFGNDLWALGCTLYQMLSGTS  
PFKDASEWLIFQRIIARDIKFPNHFSEAARDLIDRLLDTEPSRRPGAGSE  
GYVALKRHPFFNGVDWKDLRSQTPPKLAPDPASQTASPERDDTHGSPWNL  
THIGDSLATQNEGHSAPPTSSSSGSITRLASIDSFDSRWQQFLEPGESV  
30 LMISAVKKLQKITSKKVQLILTNPkLIYVDPSKLVVKGNIWSDNSNDL  
NVVVTSPSHFKICTPKKVLSFEDAKQRASVWKKAIETLQNR\*

(492 aa complete sequence.)

It will be appreciated that a further aspect of the invention is a substantially pure polypeptide comprising the above amino acid sequence or a variant, fragment,  
5 fusion or derivative thereof, or a fusion of a said variant or fragment or derivative

The said amino acid sequence may be encoded by the following nucleic acid sequence:

10

ATGTTGGCAATGGAGAAAGAATTTGATTCAAAGCTTGTTCTTCAAGGGAA  
CTCATCCAACGGTGCTAATGTTTCTAGAAGCAAAAGCTTCTCCTTTAAAG  
CTCCTCAAGAAAATTTACCAGCCATGATTTTGAATTTGGCAAGATCTAT  
GGTGTGTTGTTCTTACTCTAAGGTTGTTAGGGCAAAGAAGAAGGAACTGG  
15 AACTGTGTATGCTTTAAAGATTATGGACAAAAAGTTTATCACCAAGGAGA  
ATAAACTGCTTATGTGAAATTGGAAAGGATTGTTCTTGATCAACTTGAA  
CATCCTGGGATCATTAAACTTTACTTCACGTTTCAAGACACATCCTCACT  
ATATATGGCACTTGAATCTTGTGAGGGTGGCGAGCTTTTCGACCAAATAA  
CCAGAAAAGGTCGGCTATCGGAGGATGAAGCTCGGTTCTACACTGCAGAA  
20 GTTGTGGATGCTCTTGAGTATATACATAGTATGGGACTGATTCATCGAGA  
TATTAAGCCGGAGAATCTGTTGCTGACTTCAGATGGACACATTAAGATTG  
CGGATTTTGGAAAGTGTAAGCCGATGCAGGATAGCCAGATCACAGTTCTA  
CCTAATGCAGCTTCTGACGATAAGGCGTGCACTTTTGTCGGGACTGCTGC  
ATATGTTCTCCAGAAGTTCTCAACTCCTCTCCCGCAACTTTCGGGAATG  
25 ATCTTTGGGCTCTCGGCTGCACTCTCTATCAGATGCTTTCGGGGACTTCC  
CCATTTAAAGATGCAAGTGAATGGCTGATTTTCCAAAGAATTATAGCCAG  
AGATATAAAGTTCCCAAATCATTTTTTCAGAAGCAGCAAGAGACCTCATCG  
ACCGGTTGCTGGATACCGAGCCAAGCAGAAGGCCAGGTGCTGGCTCAGAA  
GGTTATGTTGCTCTTAAGAGACATCCTTTCTTTAATGGAGTTGACTGGAA  
30 GGATCTAAGGTCCCAGACTCCTCCAAAAGTAGCTCCAGATCCTGCGTCTC

AGACAGCATCTCCCGAGAGGGATGACACACATGGTTCTCCATGGAACCTG  
ACACATATTGGAGATTCTTTAGCCACACAGAACGAGGGGCACAGTGCTCC  
TCCTACATCTTCTGAATCATCGGGTTCCATAACTCGACTTGCTTCAATAG  
ACTCTTTTGATTCAAGATGGCAACAGTTTTTTAGAGCCAGGAGAATCGGTT  
5 CTGATGATATCAGCGGTGAAGAAGCTTCAGAAAATAACGAGCAAGAAGGT  
GCAGCTAATACTCACCAACAAACCCAAGCTGATATATGTCGACCCGTC  
AACTAGTTGTGAAAGGAAACATTATATGGTCTGATAACTCGAATGACCTC  
AACGTTGTAGTCACTAGCCCTTCACATTTCAAGATTTGCACGCCAAAGAA  
GGTTTTATCATTTGAAGACGCAAAACAGAGAGCTTCAGTGTGGAAAAAGG  
10 CAATCGAGACTCTTCAGAACCGCTGA  
(1476 bp complete sequence)

It will be appreciated that a further aspect of the invention is a recombinant polynucleotide comprising the above nucleotide sequence, or a recombinant  
15 polynucleotide encoding or suitable for expressing the above polypeptide.

A still further embodiment of the invention is a method of the invention wherein the said host yeast cell protein kinase or kinases is Ypk1 and/or Ykr2. The identification of the Ypk1 and Ykr2 genes is described in  
20 references 41 to 43.

Ypk1 from *S. cerevisiae* may comprise the following amino acid sequence:  
MYSWKSFKFGKSKEEKEAKHSGFFHSSKKEEQQNNQATAGEHDASITRS  
SLDRKGTINPSNSSVVPVRVSYDASSSTSTVRDSNGGNSSENTNSSQNLDE  
25 TANIGSTGTPNDATSSSGMMTIKVYNGDDFILPFPITSSEQILNKL LASG  
VPPPHKEISKEVDALIAQLSRVQIKNQGPADEDLISSESAAKFIPSTIML  
PGSSTLNPLLYFTIEFDNTVATIEAEYGTIAKPGFNKISTFDVTRKLPYL  
KIDVFARIPSILLPSKTWQQEMGLQDEKLQTI FDKINSNQDIHLDSFHLP  
INLSFDSAASIRLYNHHWITLDNGLGKINISIDYKPSRNKPLSIDDFDLL  
30 KVIGKGSFGKVMQVRKKDTQKVYALKAIRKSYIVSKSEVTHTLAERTVLA

RVDCPFIVPLKFSFQSPEKLYFVLAFINGGELFYHLQKEGRFDLSRARFY  
TAELLCALDNLHKLDVVYRDLKPENILLDYQGHIALCDFGLCKLNMKDDD  
KTDTFCGTPEYLAPELLLG LGYT KAVDWWTLGVLLYEMLTGLPPYYDEDV  
PKMYKKILQEPLVFPDGFDRDAKDLLIGLLSRDPTRRLGYNGADEIRNHP  
5 FFSQLSWKRLLMKGYIPPYKPAVSNSMDTSNFDEEFTREKPIDSVVDEYL  
SESVQKQFGGWTYVVGNEQLGSSMVQGRSIR

(680 aa complete sequence).

Ypk1 from *S. cerevisiae* may be encoded by the following nucleotide sequence:

10 ATGTATTCTTGGAAGTCAAAGTTTAAGTTTGGAATCTAAAGAAGAAAA  
AGAAGCCAAACATAGTGGGTTTTTTCCTCTCTAAAAGGAAGAACAGC  
AGAATAATCAAGCAACTGCGGGGGAACATGATGCTTCAATTACTCGTTCA  
TCTTTAGACAGGAAGGGCACGATAAATCCATCAAATTCATCAGTGGTCCC  
GGTTCGTGTTTCATACGATGCATCCTCATCGACCTCTACTGTACGAGATT  
15 CAAATGGTGGTAATTCAGAAAACACGAATCATCTCAAATTTAGATGAA  
ACAGCAAATATTGGTTCTACAGGGACACCCAATGATGCCACCTCAAGTTC  
AGGAATGATGACTATCAAAGTATATAATGGTGATGACTTTATTTTACCTT  
TTCCGATTACCTCAAGCGAGCAAATATTGAATAAACTATTGGCCTCGGGT  
GTTCCACCGCCACATAAGGAGATTAGTAAAGAAGTAGATGCTTTAATTGC  
20 ACAACTGTCCCGTGTTCAAATAAAGAACCAAGGTCCAGCTGATGAAGATT  
TGATCTCTAGCGAGTCAGCAGCAAAGTTCATCCCATCTACTATCATGTTA  
CCAGGGTCTTCGACCTTAAATCCCTTACTATATTTACCATTTGAATTTGA  
TAATACAGTTGCAACTATTGAAGCAGAGTATGGTACGATCGCTAAACCTG  
GTTTCAATAAGATATCTACCTTTGATGTGACAAGGAAATTACCTTATTTA  
25 AAGATTGATGTATTTGCAAGAATCCGTCCATTCTTTTGCCCTCGAAGAC  
ATGGCAACAGGAGATGGGTTTGCAAGACGAGAAGCTACAGACCATTTTGTG  
ATAAAATAAATTCCAATCAAGATATACATTTAGATTCTTTCCATTTGCCC  
ATCAACTTAAGTTTTGACTCTGCAGCTAGTATTAGACTATATAATCACCA  
CTGGATCACATTGGATAATGGCTTGGGTAAGATCAATATTAGTATTGATT  
30 ATAAACCTTCCAGAAATAAACCTTTGTCCATCGATGATTTTCGATCTTTTG

AAAGTTATCGGTAAGGGTTCGTTTGGTAAAGTGATGCAAGTCAGAAAGAA  
AGATACACAAAAAGTATACGCTTTGAAGGCAATCAGAAAATCATACATTG  
TCTCTAAATCCGAAGTCACGCATACTTTGGCAGAAAGAACCGTTCTAGCA  
CGTGTTGATTGTCCATTTATTGTACCTTTGAAGTTTTCTTTCCAATCACC  
5 GGAAAAATTATACTTTGTTTTAGCGTTTATCAATGGTGGTGAGTTGTTTT  
ATCATCTACAAAAGGAAGGAAGGTTTGATTTATCACGTGCCAGATTTTAT  
ACCGCAGAATTGTTATGTGCGCTAGACAACCTGCATAAACTAGATGTTGT  
CTATCGTGATTTGAAACCAGAGAATATTTTATTGGATTATCAAGGCCACA  
TTGCCCTTTGTGATTTCTGGGCTATGCAAATTGAATATGAAGGATGATGAT  
10 AAGACAGATACTTTTTGTGGGACCCAGAATACTTGGCACCAGAACTATT  
GCTAGGTTTAGGCTATACAAAGGCAGTAGATTGGTGGACATTGGGAGTCT  
TGTTATACGAGATGCTCACAGGTCTTCCTCCTTATTATGATGAAGATGTT  
CCAAAAATGTATAAGAAGATTCTACAGGAGCCACTAGTTTTCCAGATGG  
ATTTGATAGAGATGCAAAGGATCTATTGATTGGATTATTGAGCCGTGATC  
15 CGACAAGAAGATTGGGCTACAATGGTGCCGACGAAATTCGGAACCATCCT  
TTTTTCAGCCAATTATCATGGAAGCGCCTGTTGATGAAGGGTTATATTCC  
ACCATATAAACCAGCTGTTAGTAATTCCATGGATACTAGTAATTTGATG  
AGGAATTCAGTAGGGAGAAGCCAATTGATAGTGTGGTAGATGAATACTTG  
AGTGAAAGTGTTCAAAGCAATTTGGTGGCTGGACATACGTTGGAAATGA  
20 ACAGCTAGGTAGCTCAATGGTGCAAGGTAGAAGCATTAGATAG  
(Genbank accession no 486213; 2043 bp complete sequence)

Ykr2 (also known as Ypk2) from *S. cerevisiae* may have the following amino acid sequence:

25 MHSWRISKFKLGRSKEDDGSSSEDENEKSWGNGLFFHHGEKHHDGSPKNH  
NHEHEHHIRKINTNETLPSSLSSPKLRNDASFKNPSGIGNDNSKASERKA  
SQSSTETQGPSSSEGLMTVKVYSGKDFTLFPFITSNSTILQKLLSSGILT  
SSSNDASEVAAIMRQLPRYKRVDQDSAGEGLIDRAFATKFIPSSILLPGS  
TNSSPLLYFTIEFDNSITTISPDMGTMEQPVFNKISTFDVTRKLRFLKID  
30 VFARIPSLLLPSKNWQQEIQDEVLKEILKKINTNQDIHLDSFHLPLNL

KIDSAAQIRLYNHHWISLERGYGKLNITVDYKPSKNKPLSIDDFDLLKVI  
GKGSFGKVMQVRKKDTQKIYALKALRKAYIVSKCEVTHTLAERTVRLARVD  
CPFIVPLKFSFQSPEKLYLVLAFLINGGELFYHLQHEGRFSLARSRYIAE  
LLCALDSLHKLDVIYRDLKPENILLDYQGHIALCDFGLCKLNMKDNDKTD  
5 TFCGTPEYLAPEILLGQGYTKTVDWWTLGILLYEMMTGLPPYYDENVPVM  
YKKILQQPLLFPDGFDPAAKDLLIGLLSRDPSRRLGVNGTDEIRNHPFFK  
DISWKKLLLLKGYIPYKPIVKSEIDTANFDQEFTEKPIDSVVDEYLSAS  
IQKQFGGWYIGDEQLGDSPSQGRSIS

(677 aa complete sequence).

10

Ykr2 (also known as Ypk2) from *S. cerevisiae* may be encoded by the following nucleotide sequence:

ATGCATTCCTGGCGAATATCCAAGTTTAAGTTAGGAAGGTCCAAAGAAGA  
TGATGGGAGTAGTGAAGATGAAAATGAAAATCGTGGGGTAATGGCCTGT  
15 TTCATTTCCACCATGGAGAAAAACATCACGATGGTAGCCCGAAGAATCAT  
AATCATGAACACGAACACCATATAAGAAAGATCAATACAAATGAGACTCT  
CCCAAGTTCCTTAAGTTCTCCAAAATTACGTAATGATGCATCCTTCAAGA  
ATCCATCGGGGATAGGAAATGACAATTCTAAGGCTTCGAAAGGAAAGCT  
AGTCAGTCGTCTACTGAGACGCAGGGACCGAGTTCGGAATCCGGACTAAT  
20 GACAGTGAAGGTGTATTCTGGTAAAGATTTTACTCTTCCCTTCCCTATCA  
CCTCTAACTCTACTATTTTACAAAACTACTAAGTTCCGGCATCCTTACT  
TCATCATCCAATGACGCTTCGGAAGTTGCAGCCATAATGCGGCAGCTACC  
ACGATACAAGAGAGTGGATCAAGATTCAGCAGGGGAAGGCTTGATAGATA  
GAGCTTTTGCCACTAAATTCATTCCTTCTCTATATTGTTACCTGGGTCA  
25 ACAAATTCAAGCCATTACTTTATTTTACAATTGAATTTGATAATTCTAT  
TACTACTATTAGTCCAGATATGGGAACGATGGAGCAACCAGTGTTTAACA  
AAATATCGACATTTGATGTAACAAGAAAATTACGATTTTAAAAAATCGAT  
GTCTTTGCAAGGATTCCATCCCTACTTTTACCCTCTAAAAACTGGCAACA  
GGAGATTGGCGAGCAGGACGAAGTACTGAAGGAGATTTTAAAAAAAATCA  
30 ATACAAATCAGGATATCCATTTGGACTCCTTCCATTTACCTTTGAATTTA

AAAATCGATTCTGCAGCCCAAATAAGACTATACAATCACCATTGGATTTC  
TTTAGAAAGGGGATATGGTAAATTAAATATCACGGTGGACTACAAACCTT  
CTAAGAACAAGCCTCTCTCCATTGATGACTTTGATCTATTGAAGGTTATC  
GGGAAGGGTTCGTTTCGGCAAAGTGATGCAAGTAAGGAAAAAAGATACCCA  
5 AAAGATTTACGCTTTGAAGGCTCTGAGAAAAGCATATATTGTATCGAAAT  
GTGAAGTGACACATACTTTAGCGGAGAGGACTGTCCTAGCAAGAGTTGAC  
TGCCCCCTTTATTGTTCCGTTGAAGTTCTCATTCCAATCTCCGGAGAAGTT  
GTACCTAGTATTAGCTTTCATTAATGGCGGTGAACTGTTCTACCATTTAC  
AACACGAGGGACGATTCAGTCTAGCACGCTCCCGTTTTTTATATTGCAGAA  
10 CTATTATGTGCTCTCGATTCAATTACACAACTTGACGTCATTTATCGTGA  
CCTAAAGCCTGAAAACATTCTATTGGATTACCAAGGACATATTGCACTGT  
GTGATTTTGGGCTTTGCAAGCTGAACATGAAGGATAATGACAAAACAGAC  
ACTTTCTGTGGTACTCCCGAATATTTGGCACCAGAAATCTTGTTGGGGCA  
GGGCTATACTAAAACAGTTGACTGGTGGACATTAGGTATCTTACTGTATG  
15 AGATGATGACAGGGCTGCCACCATACTATGATGAGAACGTTCTGTTATG  
TACAAGAAAATTCTGCAGCAACCGCTACTATTTCTGATGGATTTGACCC  
TGCGGCAAAGACCTATTAATTGGCCTCTTAAGCAGAGACCCAAGCAGAA  
GACTCGGCGTTAACGGTACAGATGAAATTCGTAACCATCCTTTCTTTAAA  
GACATCTCATGGAAAAGCTACTTTTGAAGGGCTATATTCCGCCTTACAA  
20 GCCAATTGTAAAGAGTGAAATAGATACTGCAAATTTTGATCAAGAGTTCA  
CTAAGGAAAACCGATCGATAGTGTAGTGGACGAGTACTTAAGTGCAAGT  
ATTCAAAGCAGTTTGGTGGGTGGACGTACATTGGTGACGAACAGTTGGG  
TGATTCTCCTTCGCAGGGGAGAAGCATTAGTTAG

(Genbank accession no. 817862; 2034 bp complete sequence)

25

Yeast, for example *S. cerevisiae*, cells that are capable of expressing either Ypk1 or Ykr2 or both are capable of growing, but yeast, for example *S. cerevisiae*, cells that are not capable of expressing either Ypk1 or Ykr2 are not capable of growing under certain conditions, as described in Example 1.

30

A protein kinase derivable from an source other than the said host yeast cell that is equivalent to the said host yeast cell protein kinase or kinases, for example Ypk1 or Ykr2, may be serum and glucocorticoid induced protein kinase (SGK), for example mammalian, preferably human SGK. As described in Example 1, yeast, for example *S. cerevisiae*, cells that are not capable of expressing either Ypk1 or Ykr2 may be capable of growing if they are capable of expressing human SGK.

Rat SGK may have the nucleotide and amino acid sequences shown in Figures 12 and 13, respectively. The term SGK encompasses isoforms of SGK, for example SGK 1, 2 and 3 as described in Webster *et al* (1993) *Mol. Cell. Biol.* 13, 1031-2040 and Kobayashi *et al* (1999) *Biochem J* 344(Pt 1), 189-197 and in EMBL database records with Accession Nos AAD41091, AF169034 and AF169035.

PKB may also have some activity that is equivalent to Ypk1 or Ykr2 as yeast, for example *S. cerevisiae*, cells that are not capable of expressing either Ypk1 or Ykr2 may be capable of growing if they are capable of expressing high levels of PKB, ie if the cells have a high accumulation of a plasmid capable of expressing PKB, as described in Example 1. Alternatively, the ability to grow of cells that are capable of expressing PKB at a lower level may be increased by growing the cells in the presence of an activator of PKB activity.

A further aspect of the invention is a method of identifying a compound that modulates (inhibits or increases) the activity of PDK1 derivable from a first source, wherein a compound is exposed to

1) a first host yeast cell wherein the yeast cell is (a) not capable of expressing a yeast polypeptide that is a functional equivalent of human PDK1 (which may be



Pkh1 and Pkh2) and (b) is capable of expressing PDK1 derivable from the said first source

and optionally

2) a second host yeast cell wherein the yeast cell is capable of expressing a yeast polypeptide that is a functional equivalent of human PDK1 (which may be Pkh1 and/or Pkh2)

and the effect of the compound on the viability of the said yeast cell or cells is measured, and a compound that affects the viability of the first said yeast cell, or optionally that affects the viability of the first said yeast cell and the said second yeast cell differently, is identified.

A further aspect of the invention is a method of identifying a compound that modulates (inhibits or increases) the activity of a functional equivalent of Ypk1 and/or Ykr2 derivable from a first source, for example SGK or PKB, preferably human SGK or human PKB, wherein a compound is exposed to

1) a first host yeast cell wherein the yeast cell is (a) not capable of expressing a yeast polypeptide that is a functional equivalent of Ypk1 and/or Yrk2 and (b) is capable of expressing a functional equivalent of Ypk1 and/or Ykr2 (for example SGK) derivable from the said first source

and optionally

2) a second host yeast cell wherein the yeast cell is capable of expressing a yeast polypeptide (for example, an endogenous polypeptide) that is a functional equivalent of Ypk1 and/or Yrk2

and the effect of the compound on the viability of the said yeast cell or cells is measured, and a compound that affects the viability of the first said yeast cell, or optionally that affects the viability of the first said yeast cell and the said second yeast cell differently, is identified.

As in the first aspect of the invention, it will be appreciated that the said first and second host yeast cells differ substantially only in the features indicated such that apart from these features the cells are essentially the same. This ensures that there is a reasonable expectation that the effect of the compound on the phenotype of the said yeast cells can be attributed to the compound's effect on the modulation of the activity to different extents of the protein kinases as said. Thus, the first and second host yeast cells are from the same species and have substantially the same genetic content with the exception of the features indicated ie the capability to express the said host yeast cell protein kinase or kinases and the ability to express the said equivalent protein kinase derivable from a source other than the host yeast cell. It will be appreciated that the said first and second host yeast cells may differ in genetic content relating to the generation or selection of the said first or second host yeast cells (for example, in selectable marker genes used in recombinant techniques, as well known to those skilled in the art).

It will be appreciated that the PDK1 or SGK or PKB that the said first cell is capable of expressing may not be full-length PDK1 or SGK or PKB; for example, it may be a truncated PDK1 or SGK as appropriate. It will be appreciated that it is preferred that any PDK1 or SGK or PKB (either activated or not activated) that is not full length PDK1 or SGK or PKB may have at least 10, 20, 30, 40, 50, 60, 70, 80 or 90% of the enzymatic activity of full length PDK1 or SGK or PKB (either activated or not activated), as herein described.

However, it will be understood that in the methods of the invention described above it will be desirable to identify compounds that may modulate (including activate or inhibit), the activity of the relevant protein kinase or kinases, for example PDK1 or SGK, *in vivo*. Thus it will be understood that reagents and conditions used in the method may be chosen such that the interactions between

the said protein kinase and its substrate or substrates and any regulating protein/substance are substantially the same as *in vivo*.

It will be appreciated that these methods of the invention may be used to identify an activator of, for example, PDK1, SGK or PKB in a manner similar to that described above for the first and second aspects of the invention. Thus, the said first yeast cell may, under the growth conditions used in the method, express the said functional equivalent of Ypk1, Ykr2, Pkh1 and/or Pkh2 (for example, PDK1, SGK or PKB) at a level below that required for the said yeast cell to be capable of growing at the level of the wild-type cell, preferably at a level below that required for the said yeast cell to be substantially capable of growing. In the presence of an activator of the said functional equivalent (for example, PDK1, SGK or PKB), the level of activity of the said functional equivalent may be sufficient for the said yeast cell to be more capable, preferentially substantially capable of growing, under the same growth conditions. As discussed above and in Example 1, the said functional equivalent may be expressed from a regulatable promoter, for example the GAL1 promoter that is repressed in the presence of glucose.

The optional said second yeast cell may similarly be substantially unable to grow unless an activator of the said yeast polypeptide (for example, an endogenous polypeptide) is present. It will be appreciated that the yeast polypeptide may be expressed from a heterologous promoter, for example the GAL1 promoter, as discussed above and in Example 1.

It will be appreciated that when identifying compounds on the basis of the ability to increase growth (as opposed to reducing growth) of the target cell that it may be less important to make use of the optional second yeast cell in the methods above as it may be less likely that a compound that is not acting on the "target"

polypeptide (for example, the said functional equivalent of Ypk1, Ykr2, Pkh1 and/or Pkh2, which may be PDK1, SGK or PKB) would have the desired effect of increasing cell growth. Compounds acting through a "non-specific" mechanism, as discussed above, more frequently act to reduce cell growth.

5

Preferences regarding the host yeast cell and sources of the equivalent protein kinases may be as given in regard to the earlier aspects of the invention.

10

It will be appreciated that in the methods described herein, which may be drug screening methods, a term well known to those skilled in the art, the compound may be a drug-like compound or lead compound for the development of a drug-like compound.

15

20

The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than 5000 daltons and which may be water-soluble. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate target cellular membranes, but it will be appreciated that these features are not essential.

25

The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, poorly soluble, difficult to synthesise

or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

5 The compound may be suitable for use as a plant protection product or may be a lead compound for the development of a compound suitable for use as a plant protection product. It will be appreciated that such compound may have characteristics similar to those described above for drug like compounds.

It will be appreciated that compounds that may be identified by methods or screens performed with cells may be capable of interacting with or entering cells.

10

Alternatively, the methods may be used as "library screening" methods, a term well known to those skilled in the art. Thus, for example, the methods of the invention may be used to detect (and optionally identify) a polynucleotide capable of expressing a polypeptide activator of a functional equivalent of Ypk1, Ykr2, Pkh1 and/or Pkh2 for example, PDK1, SGK or PKB. Aliquots of an expression library in a suitable vector, as described below, may be tested for the ability to increase the growth of a first yeast cell that, under the growth conditions used in the method, expresses the said functional equivalent of Ypk1, Ykr2, Pkh1 and/or Pkh2 (for example, PDK1, SGK or PKB) at a level below that required for the said yeast cell to be capable of growing at the level of the wild-type cell, preferably at a level below that required for the said yeast cell to be substantially capable of growing. In the presence of a polynucleotide expressing a polypeptide activator of the said functional equivalent (for example, PDK1, SGK or PKB), the level of activity of the said functional equivalent may be sufficient for the said yeast cell to be more capable, preferentially substantially capable of growing, under the same growth conditions. As discussed above and in Example 1, the said functional equivalent may expressed from a regulatable promoter, for example the GAL1 promoter that is repressed in the presence of glucose.

15

20

25

It will be appreciated that several cycles of identifying pools of polynucleotides comprising a polynucleotide having the required property and then rescreening those polynucleotides may be required in order to identify a single species of polynucleotide with the required property.

5

It will be appreciated that further tests and/or sequence analysis may be required in order to distinguish polynucleotides encoding a functional equivalent of Ypk1, Ykr2, Pkh1 and/or Pkh2 from polynucleotides encoding an activator of a said functional equivalent.

10

Methods of introducing a polynucleotide into a host yeast cell are well known to those skilled in the art. Methods of preparing a suitable expression library for screening are well known to those skilled in the art. The library may preferably be from the same source as the said functional equivalent that is expressed in the said host yeast cell ie a human expression library may be screened for effects on yeast cells expressing human PDK1, SGK or PKB at levels below the threshold required for cell growth.

15

The above compound/library screening methods may conveniently be carried out in a 96-well microtitre plate format. A yeast cell, such as a *Saccharomyces cerevisiae* cell that has the properties of a host yeast cell for use in the method of the invention may be picked and put into liquid culture in minimal medium supplemented as necessary (as described, for example, in Example 1). The culture is grown up and may then be diluted to an optical density (OD) at 595 nm of 0.01 to 0.1. The diluted cultures are then aliquoted into wells of a sterile 96 well microtitre plate containing individual test compounds. The growth of the cells is monitored over time until the OD<sub>595</sub> reached is about ~0.8 for control cultures (ie those cultured in the absence of test compound). The OD<sub>595</sub> is assessed using a microtitre plate reader. It will be appreciated that a control

25

culture may contain an aliquot of the solvent used to dissolve the test compound. Thus, for example, the test compound may be dissolved in dimethylsulfoxide (DMSO) and both test and control cultures may therefore contain DMSO, for example at between 0.1 and 5%, preferably at about 1% to 2% by volume.

5

Alternatively, the cells may be grown on solid medium and the presence, absence, number and/or size (or other characteristic) of colonies detected. It will be appreciated that this may be performed in a high-throughput format, for example in microtitre plates. The presence, absence, number and/or size (or  
10 other characteristic) of the colonies may be detected by visual inspection, either manually or by automated techniques, including computer image analysis.

It will be appreciated that dose response measurements, as well known to those skilled in the art, may be made for selected compounds.

15

If appropriate, duplicate plates of compounds may be exposed to aliquots (including solid cultures) of a particular yeast culture, or may be exposed to aliquots of different yeast cultures. For example, one such plate may be exposed to wild-type (with respect to Pkh1, Pkh2, Ypk1, Yrk1 as appropriate) yeast cells,  
20 and a further such plate may be exposed to cells which are mutant with respect to Pkh1, Pkh2, Ypk1, Yrk2 as appropriate but which express, for example, human equivalents (PDK1 is a human equivalent of Pkh1 and Pkh2, and SGK and PKB are human equivalents of Ypk1 and Yrk2), as described in the methods of the invention.

25

#### **Yeast cells**

A further aspect of the invention is a yeast cell that is not capable of expressing Pkh1 and Pkh2 or any functional equivalent thereof. It will be appreciated that such a cell may not be capable of growing ie may be of limited viability.

- 5 A further aspect of the invention is a yeast cell that is not capable of expressing endogenous Pkh1 and/or Pkh2. The yeast cell may be capable of expressing a functional equivalent of Pkh1 and/or Pkh2 that is not the Pkh1 or Pkh2 endogenous to a wild-type yeast cell from which the said yeast cell is derived. The functional equivalent may be human PDK1 or a variant, fusion or derivative  
10 thereof, for example PDK1- $\Delta$ PH as described in Example 1. PDK1 or PDK1- $\Delta$ PH may be capable of phosphorylating the same target substrates essential for the viability of yeast cells as Pkh1 and Pkh2, as discussed in Example 1. It will be appreciated that the cell may be capable under given conditions of expressing the said functional equivalent of Pkh1 and/or Pkh2 at a level below that necessary  
15 full wild-type viability of the cell, for example at a level at which the cell is substantially unable to grow under given conditions.

- Thus, the yeast cell may be a *S. cerevisiae* cell that is not capable of expressing Pkh1 from *S. cerevisiae* or a *S. cerevisiae* cell that is not capable of expressing  
20 Pkh2 from *S. cerevisiae*, or a *S. cerevisiae* cell that is not capable of expressing Pkh1 and Pkh2 from *S. cerevisiae*. Alternatively, the yeast cell may be a cell of a different yeast species that is not capable of expressing a Pkh1 that is expressed in wild type cells of that yeast species (which may be an endogenous functional equivalent of Pkh1 from *S. cerevisiae*), or that is not capable of expressing a  
25 Pkh2 that is expressed in wild type cells of that yeast species (which may be an endogenous functional equivalent of Pkh2 from *S. cerevisiae*), or that is not capable of expressing any Pkh1 and Pkh2 (ie not capable of expressing any endogenous functional equivalent of Pkh1 and Pkh2 of *S. cerevisiae*) that are expressed in wild type cells of that yeast species.



A yeast cell not capable of expressing Pkh1 or Pkh2 or endogenous functional equivalents may be made by methods known in the art. For example, the open reading frame encoding, for example, either Pkh1 or Pkh2 may be disrupted by  
5 insertion of a selectable marker, for example as described in Example 1.

A further aspect of the invention is a yeast cell wherein one or more genes encoding a functional equivalent of PDK1, for example human PDK1, are mutated such that the yeast cell is not capable of expressing the said functional  
10 equivalent of (human) PDK1. Each such gene encoding a functional equivalent of human PDK1 may be mutated such that the yeast cell is not capable of expressing a functional equivalent of (human) PDK1. Such a functional equivalent of (human) PDK1 may be Pkh1 or Pkh2 in, for example, a *S. cerevisiae* cell.

15

It will be appreciated that cells of a yeast species other than *S. cerevisiae* may express one, two, three, four or more polypeptides that are at least partially functionally equivalent to Pkh1 and/or Pkh2 from *S. cerevisiae*. It will further be appreciated that the yeast cell of the invention encompasses a cell of such a yeast  
20 species that is not capable of expressing one, two, three, four or more or all of the said polypeptides.

It will be appreciated that Pkh1, Pkh2 and any polypeptide that is a functional equivalent of Pkh1 and/or Pkh2 may be a functional equivalent of mammalian,  
25 preferably human, PDK1. Thus, the invention encompasses a yeast cell wherein one or more gene that is present in a wild-type yeast cell that encodes a functional equivalent of human PDK1 is mutated such that the yeast cell is not capable of expressing the said functional equivalent of human PDK1. Each such gene present in a wild-type yeast cell that encodes a functional equivalent of human

PDK1 is mutated such that the yeast cell is not capable of expressing a functional equivalent of human PDK1.

5 The yeast cell of this aspect of the invention encompasses a yeast cell that is not capable of expressing a polypeptide with the sequence of Pkh1 or a functional equivalent thereof and/or Pkh2 or a functional equivalent thereof. It will be appreciated that a said yeast cell that is not capable of expressing any functional equivalent of Pkh1 or Pkh2 may not be as capable of cell growth and/or division as a wild-type yeast cell or a yeast cell that is capable of expressing a functional  
10 equivalent of Pkh1 and/or Pkh2. A functional equivalent of Pkh1 and/or Pkh2 may be capable of phosphorylating Ypk1 and/or Ykr2. PDK1 or a suitable variant, derivative, fragment or fusion thereof or a suitable fusion of a variant, derivative or fragment that is capable of phosphorylating Ypk1 and/or Ykr2 may be a functional equivalent of Pkh1 and/or Pkh2.

15

A further aspect of the invention is a said yeast cell capable of expressing a functional equivalent of Pkh1 and/or Pkh2 that is not Pkh1 or Pkh2 derivable from the wild-type yeast cell ie is an exogenous functional equivalent of Pkh1 and/or Pkh2. The said functional equivalent of Pkh1 and/or Pkh2 may be  
20 mammalian, preferably human, PDK1 or a suitable variant, derivative, fragment or fusion thereof or a suitable fusion of a variant, derivative or fragment that may be capable of phosphorylating Ypk1 and/or Ykr2. Such a yeast cell is described in Example 1. The said functional equivalent of Pkh1 or Pkh2 may be Pkh1 or Pkh2 from a different yeast species, for example a pathogenic yeast species; thus  
25 the yeast cell may be a *S. cerevisiae* cell that is (1) not capable of expressing *S. cerevisiae* Pkh1 or *S. cerevisiae* Pkh2 and (2) is capable of expressing *Candida* Pkh1 or *Candida* Pkh2.

Thus, a yeast cell of the invention may be a yeast cell wherein the wild-type gene encoding a functional equivalent of Pkh1 or Pkh2 is altered such that it is not capable of expressing a functional equivalent of the polypeptide Pkh1 or Pkh2. Thus, the yeast cell may be a *S. cerevisiae* cell wherein the polypeptide encoded  
5 by open reading frame YDR490c in wt yeast cells is not capable of being expressed and/or the polypeptide encoded by open reading frame YOL100w in wt yeast is not capable of being expressed.

The gene may be altered by the open reading frame of the gene being disrupted  
10 by insertion of a selectable marker. The marker may be *TRP1* or *HIS3*, as described in Example 1.

A further aspect of the invention is a method of identifying a compound that modulates (including increases or inhibits) the activity of PDK1, for example  
15 mammalian, preferably human, PDK1, wherein a yeast cell according to the invention is used.

A further aspect of the invention is the use of a yeast cell of the invention in a method of identifying a compound that modulates (activates or inhibits) the  
20 activity of PDK1.

By PDK1 is included mammalian, preferably human, PDK1, and a functional equivalent of PDK1 derivable from another source, preferably a source that may be pathogenic (including an opportunistic pathogen) to a mammal, preferably a  
25 human. Thus, a functional equivalent of PDK1 from a pathogenic yeast, for example *Candida* is included. Such a functional equivalent may be a polypeptide that is an equivalent of the Pkh1 or Pkh2 polypeptide identified in *S. cerevisiae*. Thus, such a functional equivalent may be the Pkh1 or Pkh2 identified in

*Candida*, as discussed in Example 4, or in the plant *Arabidopsis thaliana*, as discussed below.

A further aspect of the invention is a yeast, for example *S. cerevisiae* or *Candida*,  
5 or other yeast as listed above, cell wherein one or more endogenous gene(s) encoding a functional equivalent of human SGK or PKB is mutated such that the yeast cell is not capable of expressing the said functional equivalent of human SGK or PKB. The said gene may be the Ypk1 or Yrk2 gene. Each such endogenous gene encoding a functional equivalent of human SGK or PKB may be  
10 mutated such that the yeast cell is not capable of expressing an endogenous functional equivalent of, for example, human SGK or PKB.

A further aspect of the invention is a yeast, for example *S. cerevisiae* or *Candida*, or other yeast/fungus, as listed above, cell wherein one or more endogenous  
15 genes encoding a functional equivalent of human SGK is mutated such that the yeast cell is not capable of expressing the said functional equivalent of human SGK.

As discussed in Example 4, the following nucleotide sequences may encode  
20 polypeptides related to *S. cerevisiae* Ypk1 and Yrk2 polypeptides: 384362E11.s1.seq and 384286E10.s1.seq.

The said gene may be a gene from which Ypk1 may be expressed or a gene from which Ykr2 may be expressed.

25

A further aspect of the invention is a said yeast cell wherein each such endogenous gene encoding a functional equivalent of human SGK or Ypk1 or Yrk2 is mutated such that the yeast cell is not capable of expressing an endogenous functional equivalent of, for example, human SGK or Ypk1 or Yrk2.

30

A further aspect of the invention is a method of identifying a compound (including a polypeptide or polynucleotide encoding a polypeptide) that modulates (activates or inhibits) the activity of SGK, for example fungal or mammalian, preferably human, SGK, wherein a yeast cell according to the above aspect of the invention is used.

A further aspect of the invention is the use of a yeast cell according to the above aspect of the invention in a method of identifying a compound that modulates (activates or inhibits) the activity of SGK.

#### Ypk1 and Ykr2

A further aspect of the invention is a protein kinase derivable from yeast capable of phosphorylating a polypeptide comprising the consensus sequence Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr-Hyd, for example the polypeptide Crosstide (which has the amino acid sequence GRPRTSSFAEG) or polypeptide 2, 8 or 11 shown in Table 2 of Example 1.

A further aspect of the invention is a protein kinase derivable from yeast capable of being phosphorylated by Pkh1 or Pkh2 or mammalian, preferably human, PDK1.

The yeast may be *S. cerevisiae*. The protein kinase may share functional characteristics with mammalian PKB $\alpha$  and/or SGK. It was not previously known that yeast, for example *S. cerevisiae*, may have a protein kinase with similar functional properties to mammalian PKB $\alpha$  or SGK.

The protein kinase may be Ypk1 from *S. cerevisiae* or equivalent open reading frame in yeast other than *S. cerevisiae*, for example *Candida* spp or Ykr2 from *S.*

*cerevisiae* or equivalent open reading frame in yeast other than *S. cerevisiae*, for example *Candida* spp. Alternatively and less preferably, it may be a related polypeptide pkc1 or sch9 or other AGC (protein kinase A/protein kinase G/protein kinase C) family member.

5

A further aspect of the invention is a variant, derivative, fragment or fusion thereof or a fusion of a variant, derivative or fragment of a protein kinase according to the previous aspect of the invention that is capable of being phosphorylated by Pkh1 or Pkh2 or mammalian, preferably human, PDK1 and/or

10 capable of phosphorylating a polypeptide comprising the consensus sequence Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr-Hyd.

The protein kinase may be the polypeptide encoded by the *YPK1* gene [41] or the *YKR2/YPK2* gene [42].

15

#### **Pkh1 and Pkh2**

A further aspect of the invention is a substantially pure polypeptide encoded by open reading frame YDR490c of *S. cerevisiae* or equivalent open reading frame

20 in yeast other than *S. cerevisiae* or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative

or

a substantially pure polypeptide encoded by open reading frame YOL100w of *S. cerevisiae* or equivalent open reading frame in yeast other than *S. cerevisiae*.

25 or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative wherein the polypeptide does not comprise the amino acid sequence of human PDK1 or *Drosophila* PDK1 (DSTPK61).

The said polypeptides are considered to be protein kinases, in particular protein kinases that are capable of phosphorylating PKB $\alpha$ , particularly human PKB $\alpha$ , in particular on residue Thr308. They are further considered to be capable of phosphorylating the yeast polypeptides known as Ypk1 and Ykr2, as described

5 above, human SGK or a polypeptide with the consensus sequence Thr-Phe-Cys-Gly-Thr-X-Glu-Tyr (which may be present with the "activation loop" of the catalytic domain between conserved subdomains VII and VIII of a protein kinase).

10 The polypeptides described above are herein referred to as Pkh1 (PKB-activating kinase homologue 1; encoded by open reading frame YDR490c of *S. cerevisiae*, for example) or Pkh2 (PKB-activating kinase homologue 2; encoded by open reading frame YOL100w of *S. cerevisiae*, for example) [42, 43].

15 Partial amino acid sequences of Pkh1 and Pkh2 are also shown in Figure 1.

Example 4 describes the identification of *Candida albicans* nucleotide sequences that may encode polypeptides related to *S. cerevisiae* Pkh1 and Pkh2. The method given in Example 4 may be applicable to identifying related open reading

20 frames in other organisms, for example other yeasts.

The following nucleotide sequences may encode polypeptides related to *S. cerevisiae* Pkh1 and Pkh2: 384194F08.s1.seq and 396076E03.s2.seq

25 By "substantially pure" we mean that the said polypeptide is substantially free of other proteins. Thus, we include any composition that includes at least 30% of the protein content by weight as the said polypeptide, preferably at least 50%, more preferably at least 70%, still more preferably at least 90% and most preferably at least 95% of the protein content is the said polypeptide.

Thus, the invention also includes compositions comprising the said polypeptide and a contaminant wherein the contaminant comprises less than 70% of the composition by weight, preferably less than 50% of the composition, more preferably less than 30% of the composition, still more preferably less than 10% of the composition and most preferably less than 5% of the composition by weight.

The invention also includes the substantially pure said polypeptide when combined with other components *ex vivo*, said other components not being all of the components found in the cell in which said polypeptide is found. It will be appreciated that the said substantially pure polypeptide may be obtained by expression from a recombinant nucleic acid, for example in a prokaryotic or eukaryotic cell, as discussed further below.

15

Variants (whether naturally-occurring or otherwise) may be made using the methods of protein engineering and site-directed mutagenesis well known in the art using the recombinant polynucleotides described below.

20 By "fragment of said polypeptide" we include any fragment which retains activity, for example a protein kinase activity, for example as described above, or which is useful in some other way, for example, for use in raising antibodies or in a binding assay.

25 By "fusion of said polypeptide" we include said polypeptide fused to any other polypeptide. For example, the said polypeptide may be fused to a polypeptide such as glutathione-S-transferase (GST) or protein A in order to facilitate purification of said polypeptide. Examples of such fusions to GST are given in Example 1. Similarly, the said polypeptide may be fused to an oligo-histidine tag



such as His<sub>6</sub> or to an epitope recognised by an antibody such as the well known Myc tag epitope. Fusions to any variant, fragment or derivative of said polypeptide are also included in the scope of the invention.

- 5 By “variants” of the polypeptide we include insertions, deletions and substitutions, either conservative or non-conservative. In particular we include variants of the polypeptide where such changes do not substantially alter the activity of the said polypeptide (for example, the ability to phosphorylate PKB $\alpha$ , particularly human PKB $\alpha$ , in particular on residue Thr308). Variants of Pkh1 or  
10 Pkh2 do not include polypeptides which have the amino acid sequence of *Schizosaccharomyces pombe*, human PDK1 or *Drosophila* PDK1 (DSTPK61); see Figure 1.

It will be appreciated that a variant that comprises substantially full-length Pkh1  
15 or Pkh2 may be particularly useful. By “substantially full-length” is meant comprising at least 80%, preferably 90%, still more preferably 95%, 98% or 100% (ie all) of the sequence of the full length polypeptide.

By “conservative substitutions” is intended combinations such as Gly, Ala; Val,  
20 Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

It is particularly preferred if the polypeptide variant has an amino acid sequence which has at least 65% identity with the amino acid sequence given above, more preferably at least 75%, still more preferably at least 80%, yet more preferably at  
25 least 90%, and most preferably at least 95% or 99% identity with the amino acid sequence given above.

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of

Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequences have been aligned optimally.

- 5 The alignment may alternatively be carried out using the Clustal W program (Thompson *et al* (1994) *Nucl Acid Res* 22, 4673-4680). The parameters used may be as follows:

Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

- 10 Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

Scoring matrix: BLOSUM.

- 15 Thus, using these parameters, the catalytic domain of Pkh1 and Pkh2 may have 50% and 49% identity respectively with the catalytic domain of human PDK1 and 41% identity with the catalytic domain of *Drosophila* PDK1. There appears to be no significant identity between the non-catalytic domains of human or *Drosophila* PDK1 and either Pkh1 or Pkh2.

- 20 A particular embodiment of the invention provides a substantially pure yeast Pkh1 polypeptide or naturally occurring allelic variants thereof. A partial amino acid sequence is also shown in Figure 1.

- 25 A further particular embodiment of the invention provides a substantially pure Pkh2 polypeptide or naturally occurring allelic variants thereof.

It is particularly preferred, although not essential, that the variant or fragment or derivative or fusion of the said polypeptide, or the fusion of the variant or fragment or derivative has at least 30% of the enzyme activity of Pkh1 or Pkh2,

respectively, with respect to the phosphorylation (or activation) of PKB $\alpha$  (particularly human PKB $\alpha$ ) or other polypeptide comprising the consensus sequence B-T-F-C-G-T-P/I-D/E-Y-L/I/M-A-P-E, for example a protein kinase belonging to the AGC subfamily (protein kinase A/G/C subfamily), in particular  
5 SGK, p70 S6 Kinase or PKC $\zeta$ , as discussed in Example 1 and shown in Figure 6. It is more preferred if the variant or fragment or derivative or fusion of the said polypeptide, or the fusion of the variant or fragment or derivative has at least 50%, preferably at least 70% and more preferably at least 90% of the enzyme activity of Pkh1 or Pkh2 with respect to the phosphorylation of PKB $\alpha$  or any one  
10 of the alternatives described above. However, it will be appreciated that variants or fusions or derivatives or fragments which are devoid of enzymatic activity may nevertheless be useful, for example by interacting with another polypeptide, or as antigens in raising antibodies.

15 A further aspect of the invention provides a recombinant polynucleotide encoding a said polypeptide of the invention (for example, Pkh1 or Pkh2 from *S. cerevisiae*) or encoding a variant or fragment or derivative or fusion of said polypeptide or a fusion of a said variant or fragment or derivative. Preferences and exclusions for the said polynucleotide variant are the same as given above.

20 A further aspect of the invention provides a recombinant polynucleotide suitable for expressing a polypeptide of the invention or suitable for expressing a variant or fragment or derivative or fusion of said polypeptide or a fusion of a said variant or fragment or derivative. Preferences and exclusions for the said  
25 polynucleotide variant are the same as in the first aspect of the invention.

By "suitable for expressing" is mean that the polynucleotide is a polynucleotide that may be translated to form the polypeptide, for example RNA, or that the polynucleotide (which is preferably DNA) encoding the polypeptide of the

invention is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The polynucleotide may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by any desired host; such controls may be  
5 incorporated in the expression vector.

Thus a further aspect of the invention is a replicable vector suitable for expressing a polypeptide of the invention or suitable for expressing a variant or fragment or derivative of fusion of said polypeptide or a fusion of a said variant  
10 or fragment or derivative. Preferences and exclusions for the said polynucleotide variant are the same as for the polypeptide of the invention. For example, the replicable vector may be suitable for expressing a fusion of the polypeptide of the invention, in particular a GST fusion, for example as described in Example 1. It will be appreciated that a construct is not a recombinant polynucleotide as defined  
15 above if it lacks sequences necessary for the translation and therefore expression of the encoded polypeptide of the invention.

A further aspect of the invention is a polynucleotide encoding a fusion of the polypeptide of the invention, or a fusion of a variant or fragment or derivative, in  
20 particular a GST fusion. A still further aspect is a vector suitable for replication in a mammalian/eukaryotic cell, comprising a polynucleotide encoding the polypeptide, or a variant or fragment or derivative or a fusion of the polypeptide, as defined in the first aspect of the invention, or a fusion of a variant or fragment or derivative, in particular a GST fusion.

25

Characteristics of vectors suitable for replication in mammalian/eukaryotic cells are well known to those skilled in the art, and examples are given below. It will be appreciated that a vector may be suitable for replication in both prokaryotic and eukaryotic cells.

A polynucleotide comprising a fragment of the recombinant polynucleotide encoding a polypeptide of the invention or a variant, fragment, fusion or derivative may also be useful. Preferably, the polynucleotide comprises a  
5 fragment which is at least 10 nucleotides in length, more preferably at least 14 nucleotides in length and still more preferably at least 18 nucleotides in length. Such polynucleotides are useful as PCR primers. A polynucleotide complementary to the polynucleotide (or a fragment thereof) encoding a polypeptide of the invention or a variant, fragment, fusion or derivative may also  
10 be useful. Such complementary polynucleotides are well known to those skilled in the art as antisense polynucleotides.

The polynucleotide or recombinant polynucleotide of the invention may be DNA or RNA, preferably DNA. The polynucleotide may or may not contain introns in  
15 the coding sequence; preferably the polynucleotide is a cDNA.

A "variation" of the polynucleotide includes one which is (i) usable to produce a protein or a fragment thereof which is in turn usable to prepare antibodies which specifically bind to the protein encoded by the said polynucleotide or (ii) an  
20 antisense sequence corresponding to the gene or to a variation of type (i) as just defined. For example, different codons can be substituted which code for the same amino acid(s) as the original codons. Alternatively, the substitute codons may code for a different amino acid that will not affect the activity or immunogenicity of the protein or which may improve or otherwise modulate its  
25 activity or immunogenicity. For example, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, "Strategies and Applications of *In Vitro* Mutagenesis" *Science*, 229: 193-210 (1985), which is incorporated herein by reference. Since such modified

polynucleotides can be obtained by the application of known techniques to the teachings contained herein, such modified polynucleotides are within the scope of the claimed invention.

- 5 Moreover, it will be recognised by those skilled in the art that the polynucleotide sequence (or fragments thereof) encoding a polypeptide of the invention can be used to obtain other polynucleotide sequences that hybridise with it under conditions of high stringency. Such polynucleotides includes any genomic DNA. Accordingly, the polynucleotide of the invention includes polynucleotide that
- 10 shows at least 60%, preferably 70%, and more preferably at least 80% and most preferably at least 90% homology with the polynucleotide identified in the method of the invention, provided that such homologous polynucleotide encodes a polypeptide which is usable in at least some of the methods described below or is otherwise useful. Such a polypeptide may be a functional homologue of the
- 15 polypeptide of the invention. The polypeptide may, for example, have a similar enzymatic activity to the polypeptide of the invention or may be able to substitute for the polypeptide of the invention in a cell, as discussed above.

It will be appreciated that such a method may be used in the identification of a

20 functional homologue of Ypk1 or Ykr2, as discussed above.

It is preferred that the polynucleotide is derivable from a yeast, for example a yeast other than *S. cerevisiae* or *Candida*. It is particularly preferred that the polynucleotide is derivable from a pathogenic yeast or a yeast that may be useful

25 as the host cell in a screening assay as described below.

Per cent homology can be determined by, for example, the GAP program of the University of Wisconsin Genetic Computer Group.

DNA-DNA, DNA-RNA and RNA-RNA hybridisation may be performed in aqueous solution containing between 0.1XSSC and 6XSSC and at temperatures of between 55°C and 70°C. It is well known in the art that the higher the temperature or the lower the SSC concentration the more stringent the hybridisation conditions. By "high stringency" we mean 2XSSC and 65°C. 1XSSC is 0.15M NaCl/0.015M sodium citrate. Polynucleotides which hybridise at high stringency are included within the scope of the claimed invention.

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* 69, 2110 and

Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* 275, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

- 10 Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cell, bacterial cells, insect cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25:FD.

- 20 Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* 194, 182.

Methods and vectors for transformation of *Candida* spp are described in, for example, Zhou P, et al "A system for gene cloning and manipulation in the yeast *Candida glabrata*." *Gene*. 1994 May 3;142(1):135-40; Mehra RK, et al "Cloning system for *Candida glabrata* using elements from the metallothionein-IIa-encoding gene that confer autonomous replication." *Gene*. 1992 Apr 1;113(1):119-24; Ohkuma M, et al "Cloning of the C-URA3 gene and construction of a triple auxotroph (his5, ade1, ura3) as a useful host for the



- genetic engineering of *Candida maltosa*." Curr Genet. 1993 Mar;23(3):205-10; Sakai Y, et al "Transformation system for an asporogenous methylotrophic yeast, *Candida boidinii*: cloning of the orotidine-5'-phosphate decarboxylase gene (URA3), isolation of uracil auxotrophic mutants, and use of the mutants for integrative transformation." J Bacteriol. 1991 Dec;173(23):7458-63; Haas LO, et al "Development of an integrative DNA transformation system for the yeast *Candida tropicalis*." J Bacteriol. 1990 Aug;172(8):4571-7; Sakai Y, et al "High-frequency transformation of a methylotrophic yeast, *Candida boidinii*, with autonomously replicating plasmids which are also functional in *Saccharomyces cerevisiae*." J Bacteriol. 1993 Jun;175(11):3556-62; Kitada K, et al "Cloning of the *Candida glabrata* TRP1 and HIS3 genes, and construction of their disruptant strains by sequential integrative transformation." Gene. 1995 Nov 20;165(2):203-6; Macreadie IG, et al "Heterologous gene expression and protein secretion from *Candida glabrata*." Biotechnol Appl Biochem. 1994 Jun;19 ( Pt 3):265-9. Hsu WH, et al "Construction of a new yeast cloning vector containing autonomous replication sequences from *Candida utilis*." J Bacteriol. 1983 Jun;154(3):1033-9; Hanic-Joyce PJ, et al "A high-copy-number ADE2-bearing plasmid for transformation of *Candida glabrata*." Gene. 1998 May 12;211(2):395-400.
- 20 Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the
- 25 DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* 98, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce  
5 proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally  
10 homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

A further aspect of the invention provides a method of making the polypeptide of the invention or a variant, derivative, fragment or fusion thereof or a fusion of a  
15 variant, fragment or derivative the method comprising culturing a host cell comprising a recombinant polynucleotide or a replicable vector which encodes said polypeptide, and isolating said polypeptide or a variant, derivative, fragment or fusion thereof or a fusion of a variant, fragment or derivative from said host cell. Methods of cultivating host cells and isolating recombinant proteins are well  
20 known in the art.

The invention also includes a polypeptide, or a variant, fragment, derivative or fusion thereof, or fusion of a said variant or fragment or derivative obtainable by the above method of the invention.

25

A still further aspect of the invention provides an antibody reactive towards a polypeptide of the invention. Examples of such antibodies and of methods of preparing such antibodies are given in Example 1.

Antibodies reactive towards the said polypeptide of the invention may be made by methods well known in the art. In particular, the antibodies may be polyclonal or monoclonal.

- 5    Suitable monoclonal antibodies which are reactive towards the said polypeptide may be prepared by known techniques, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", SGR Hurrell (CRC Press, 1982).

10

In a preferred embodiment the antibody is raised using any suitable peptide sequence obtainable from the amino acid sequence of Pkh1 or Pkh2 as appropriate. It is preferred if polyclonal antipeptide antibodies are made.

- 15    It is particularly preferred if the antibody does not react substantially with another protein kinase, in particular another yeast protein kinase. Accordingly, it may be preferred if peptides based on the Pkh1 or Pkh2 sequence are used which vary significantly from any peptides found in any other protein kinase, particularly a yeast protein kinase. It may also be preferred that an antibody reacts with Pkh1  
20    but does not react substantially with Pkh2, and *vice versa*.

Techniques for preparing antibodies are well known to those skilled in the art, for example as described in Harlow, ED & Lane, D "Antibodies: a laboratory manual" (1988) New York Cold Spring Harbor Laboratory.

25

### Other methods

A further aspect of the invention provides a method of identifying a drug-like compound or lead compound for the development of a drug-like compound that

modulates the activity of the polypeptide Pkh1, Pkh2, Ypk1 or Ykr2, the method comprising contacting a compound with the polypeptide or a suitable variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof and determining whether the protein kinase activity of the said  
5 polypeptide is changed compared to the activity of the said polypeptide or said variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof in the absence of said compound.

The terms “drug-like compound” and “lead compound” are well known to those  
10 skilled in the art, as discussed above.

The compound may act by interacting with the said polypeptide and modulating, for example inhibiting, its activation by an “upstream activator”. The compound may act by interacting with Ypk1 or Ykr2 and modulating, for example  
15 inhibiting, its activation by an “upstream activator”, for example Pkh1 or Pkh2.

It will be understood that it will be desirable to identify compounds that may modulate the activity of the polypeptide *in vivo*. Thus it will be understood that reagents and conditions used in the method may be chosen such that the  
20 interactions between the said polypeptide and its substrate are substantially the same as between yeast, for example *S. cerevisiae* Pkh1, Pkh2, Ypk1 or Ykr2 and their substrate or substrates *in vivo*. An example of a substrate of said Pkh1 or Pkh2 polypeptide is Ypk1 or Ykr2.

25 In one embodiment, the compound decreases the activity of said polypeptide. For example, the compound may bind substantially reversibly or substantially irreversibly to the active site of said polypeptide. In a further example, the compound may bind to a portion of said polypeptide that is not the active site so as to interfere with the binding of the said polypeptide to its substrate. In a still

further example, the compound may bind to a portion of said polypeptide so as to decrease said polypeptide's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said polypeptide's activity, for example in the activation of the said polypeptide by an  
5 "upstream activator", such as Pkh1 or Pkh2 for Ypk1 and Ykr2.

In a further embodiment, the compound increases the activity of said polypeptide. For example, the compound may bind to a portion of said polypeptide that is not the active site so as to aid the binding of the said polypeptide to its substrate. In  
10 a still further example, the compound may bind to a portion of said polypeptide so as to increase said polypeptide's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said polypeptide's activity for example in the activation of the said polypeptide by an "upstream activator", such as Pkh1 or Pkh2 for Ypk1 and Ykr2.

15

Conveniently, the method makes use of the fact that Pkh1 or Pkh2 phosphorylates, for example Ypk1 or Ykr2 as described in Example 1, or that Ypk1 or Ykr2 phosphorylate peptides as shown in Table 2 and discussed in Example 1, but any suitable substrate may be used. Thus the phosphorylation of  
20 Ypk1 or Ykr2 or peptide substrate of Ypk1 or Ykr2 may be measured using techniques well known to those skilled in the art. Conveniently, the method makes use of an assay which may be substantially the same as that described in Example 1 or which may be adapted to render the assay more convenient for high-throughput screening, as well known to those skilled in the art. For  
25 example, a scintillation proximity assay (SPA; Amersham) may be useful. It is preferred that the polypeptide (for example, Pkh1, Pkh2, Ypk1 or Ykr2) is recombinant. It is preferred that the substrate is recombinant or synthetic.

Alternatively, a change in the activity of the substrate may be measured. For example, the protein kinase activity of Ypk1 or Ykr2 or a substrate of Ypk1 or Ykr2 may be measured, as described above. This may be done in a whole cell system or using purified or partially purified components. Expression of an  
5 protein encoded by an RNA transcribed from a promoter regulated by a substrate of Ypk1 or Ykr2 may be measured. The protein may be one that is physiologically regulated by a substrate of Ypk1 or Ykr2 or may be a "reporter" protein, as well known to those skilled in the art (ie a recombinant construct may be used). A reporter protein may be one whose activity may easily be assayed,  
10 for example  $\beta$ -galactosidase, chloramphenicol acetyltransferase or luciferase (see, for example, Tan *et al* (1996)).

A further aspect of the invention is a method of identifying a compound which modulates, for example blocks, the activation of a polypeptide that is a functional  
15 equivalent of Ypk1 and/or Ykr2 that is not SGK, PKB $\alpha$  or p70S6 kinase (or is derivable from a non-mammalian source) by an interacting polypeptide, for example Pkh1, Pkh2 or PDK1, the method comprising determining whether a compound enhances or disrupts the interaction between (a) a polypeptide that is a functional equivalent of Ypk1 and/or Ykr2 that is not SGK, PKB $\alpha$  or p70S6  
20 kinase (or is derivable from a non-mammalian source) or a suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative and (b) the interacting polypeptide, or a suitable variant, derivative, fragment or fusion thereof or a suitable fusion of a variant, derivative or fragment, or determining whether the compound substantially blocks activation of  
25 the said polypeptide that is a functional equivalent of Ypk1 and/or Ykr2 that is not SGK, PKB $\alpha$  or p70S6 kinase (or is derivable from a non-mammalian source) or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion by the interacting polypeptide, or a suitable variant, derivative, fragment or fusion thereof.

It will be understood that it will be desirable to identify compounds that may modulate the activity of the polypeptide *in vivo*. Thus it will be understood that reagents and conditions used in the method may be chosen such that the interactions between the said polypeptide that is a functional equivalent of Ypk1 and/or Ykr2 that is not SGK, PKB $\alpha$  or p70S6 kinase (or is derivable from a non-mammalian source) and the interacting polypeptide, for example Pkh1, Pkh2, PDK1 and/or a protein kinase with PDK2 activity are substantially the same as between a said naturally occurring polypeptide that is a functional equivalent of Ypk1 and/or Ykr2 and a naturally occurring interacting polypeptide *in vivo*. PDK1 may be a polypeptide with PDK2 activity, for example when bound to PIF or a related polypeptide, as discussed above.

It will be appreciated that the said suitable variant, fragment, derivative or fusion of a polypeptide that is a functional equivalent of Ypk1 and/or Ykr2 that is not SGK, PKB $\alpha$  or p70S6 kinase (or is derivable from a non-mammalian source), or a fusion of a said fragment, derivative or fusion is not SGK, PKB $\alpha$  or p70S6 kinase.

A further aspect of the invention is the use of Pkh1 or Pkh2 or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion thereof that is not PDK1 to phosphorylate and/or activate a polypeptide that is Ypk1 and/or Ykr2 or SGK or PKB $\alpha$  or a functional equivalent thereof or suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion.

A further aspect of the invention is the use of PDK1 or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion thereof to phosphorylate and/or activate a polypeptide that is Ypk1

and/or Ykr2 or SGK or a functional equivalent thereof that is not PKB $\alpha$  or p70S6 kinase or suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion.

- 5 A further aspect of the invention is a method of identifying a compound which binds to Ypk1 or Ykr2 or SGK (or other substrate of Pkh1, Pkh2 or PDK1) and either enhances or prevents its activation by Pkh1, Pkh2 or PDK1, the method comprising determining whether a compound enhances or prevents the interaction of Ypk1 or Ykr2 or SGK (or other substrate of Pkh1, Pkh2 or PDK1) or a  
10 suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative with Pkh1, Pkh2 or PDK1 or a suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative or determining whether the compound substantially blocks activation of Ypk1 or Ykr2 or SGK (or other substrate Pkh1, Pkh2 or PDK1) or a suitable  
15 fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative by Pkh1, Pkh2 or PDK1.

Suitable assays may be similar to those described above.

- 20 A further aspect of the invention is a method of identifying a polypeptide that interacts with Pkh1 or Pkh2 or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion thereof that is not PDK1, the method comprising 1) contacting a) the said polypeptide with b) a composition that may contain such an interacting polypeptide, 2) detecting the  
25 presence of a complex containing the said polypeptide and an interacting polypeptide, and optionally 3) identifying any interacting polypeptide bound to the said polypeptide.



In one embodiment, the composition may comprise material from cells. In particular, the cells may be selected from the following types: (1) cells which have Pkh1 or Pkh2 activity after exposure to a stimulus, but which have not been so exposed and (2) cells of type 1 after exposure to the stimulus. Polypeptides that are found in one only of types 1 or 2 are of particular interest and may be characterised further. Such a peptide may be an activator of Pkh1 or Pkh2. Alternatively, it may be an inactivator of Pkh1 or Pkh2.

It will be appreciated that the method may be performed within a cell, for example using the yeast two hybrid system as is well known in the art. In this example, cDNAs copied from mRNA from the two cell types described above would be used.

It will further be appreciated that a recombinant yeast cell in which a Pkh1 or Pkh2 gene is altered and/or a recombinant polynucleotide capable of expressing Pkh1 or Pkh2 is present, may be useful in, for example, identifying a substrate of Pkh1 or Pkh2, as described in Example 1.

A further aspect of the invention is a polypeptide identifiable by the said method.

A still further aspect of the invention provides a method of identifying a compound which modulates the activation of Pkh1 or Pkh2 or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion thereof that is not PDK1 by an "upstream activator". By "upstream activator" is meant a molecule that interacts with the polypeptide of the invention with the result that the protein kinase activity of the polypeptide of the invention is increased. It may be a polypeptide. Preferably, it is a physiological activator of native Pkh1 or Pkh2. Such an activator may be identified by the method given above.

A further aspect of the invention is the use of Pkh1 or Pkh2 or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion thereof for the activation of Ypk1 or Ykr2 or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion that is not SGK or PKB $\alpha$ .

A further aspect of the invention is a method of identifying a compound which blocks the activation of Pkh1 or Pkh2 or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion thereof by a said interacting polypeptide identifiable by the above method, the method comprising determining whether a compound enhances or disrupts the interaction between (a) the said Pkh1 or Pkh2 and (b) said interacting polypeptide or a suitable variant, derivative, fragment or fusion thereof or a suitable fusion of a variant, derivative or fragment, or determining whether the compound substantially blocks activation of the said polypeptide as defined in the first aspect of the invention by said interacting polypeptide or a suitable variant, derivative, fragment or fusion thereof.

It will be appreciated that screening assays which are capable of high throughput operation will be particularly preferred. Examples may include cell based assays and protein-protein binding assays. An SPA-based (Scintillation Proximity Assay; Amersham International) system may be used. For example, beads comprising scintillant and a polypeptide that may be phosphorylated may be prepared. The beads may be mixed with a sample comprising the protein kinase, as described above and  $^{32}\text{P}$ -ATP or  $^{33}\text{P}$ -ATP and with the test compound. Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known parameters for  $^{32}\text{P}$  or  $^{33}\text{P}$  SPA assays. Only  $^{32}\text{P}$  or  $^{33}\text{P}$  that is in proximity to the scintillant, i.e. only that bound to the

polypeptide, is detected. Variants of such an assay, for example in which the polypeptide is immobilised on the scintillant beads *via* binding to an antibody, may also be used.

- 5 Other methods of detecting polypeptide/polypeptide interactions include ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Fluorescence Energy Resonance Transfer (FRET) methods, for example, well known to those skilled in the art, may be used, in which binding of two fluorescent labelled entities may be measured by measuring  
10 the interaction of the fluorescent labels when in close proximity to each other.

### Medical aspects

- A further aspect of the invention is a compound identified or identifiable by any  
15 appropriate one of the above methods of the invention.

A still further aspect of the invention is a said compound for use in medicine.

- The compound may be a compound that inhibits the activity mammalian,  
20 preferably human PDK1 or mammalian, preferably human SGK. Alternatively, the compound may inhibit a functional homologue of PDK1 or SGK in a pathogen or opportunistic pathogen, for example of a mammal, preferably a human. For example, the compound may inhibit a functional homologue of PDK1 (which may be Pkh1 or Pkh2) or SGK (which may be Ypk1 or Yrk2) in  
25 *Candida* species or any other pathogenic yeast species as listed above. Such a compound may be useful as an antifungal agent.

It will be appreciated that the pathogen or opportunistic pathogen may affect a plant, which may be a monocotyledenous plant or a dicotyledenous plant. For

example, the pathogen may be a member of the Uredinales group of fungi that may cause the plant disease rust, for example *Puccinia graminis* which may cause black rust of cereals, or may be *Phytophthora infestans* which may cause potato blight. Thus, a compound that may inhibit a functional homologue of PDK1  
5 (which may be Pkh1 or Pkh2) or SGK (which may be Ypk1 or Yrk2) in a yeast that is pathogenic for a plant may be useful as an antifungal agent for use as a plant protection product ie for treating or preventing a fungal infection of a plant.

It will be appreciated that a compound that inhibits mammalian, for example  
10 human PDK1 may be useful in medicine. Thus, a further aspect of the invention is a method of modulating in a cell the activity of PDK1 (which may include its interactions with protein kinase B or SGK), the method comprising exposing the cell to a compound of the invention. It will be appreciated that the method may be used *in vitro* or *in vivo*. It is preferred that the compound of the invention is  
15 capable of entering the cell and that it enters the cell.

A still further aspect of the invention is a method of treating a patient in need of modulation, preferably inhibition, of the activity of PDK1 or its interactions with protein kinase B the method comprising administering to the patient an effective  
20 amount of a compound of the invention that inhibits mammalian, for example human, PDK1.

Compounds identifiable in the screening methods of the invention that inhibit PKB, PDK1 or the activation of PKB by PDK1 are believed to be useful in  
25 treating cancer. PKB is the cellular homologue of v-akt which is involved in leukaemias. Two isoforms of PKB are overexpressed in ovarian, pancreatic and breast cancers. It is believed that PKB mediates protection of cells to apoptosis mediated, for example, by IGF-1. Overexpression of PKB may allow cancer cells to proliferate by stopping apoptosis. Promotion of apoptosis may be

beneficial in the resolution of inflammation.

A compound that results in activation of PKB or PDK1 may be useful in the treatment of diabetes or obesity. Such a compound may also be useful in the treatment of patient before, after or during heart surgery. Such a compound may also be useful in reducing apoptosis; thus, such a compound may be useful in treating a patient in need of protection against apoptosis. Reducing apoptosis may be useful following ischaemic injury, for example stroke or myocardial infarction, and in tissue repair.

10

A compound of the invention that inhibits a fungal functional homologue of PDK1 (which may be Pkh1 or Pkh2) or SGK (which may be Ypk1 or Yrk2) may be useful in medicine, for example as an antifungal agent. It may be useful in treating a *Candida* infection, for example thrush. Thus, a further aspect of the invention is the use of a compound of the invention that inhibits a fungal functional homologue of PDK1 or SGK in the manufacture of a medicament for treating or preventing a fungal infection, for example thrush.

15

Further examples of fungal infections that may be treated by a compound of the invention include infections that may be caused by a pathogenic fungus listed above. Still further examples of infections or conditions that may be treated or prevented by a compound of the invention are listed below. It will be appreciated that it may be preferred that the compound of the invention has been selected, for example using a method of the invention as described above, for an effect on a protein kinase of the yeast genus or species indicated.

20

25

Thus, a compound of the invention may be used in the manufacture of a medicament for the treatment or prevention of an infections that may be caused by a pathogenic fungus listed above or an infection or condition listed below.

Aspergillosis may be caused by fungi of the genus *Aspergillus*, usually *A. fumigatus*. Blastomycosis is caused by the fungus *Blastomyces dermatitidis*. Infection may take place through the lungs but the infection may become widely  
5 disseminated, with the skin, skeleton and genito-urinary system becoming infected.

Candidiasis is caused by *Candida* spp, particularly *C. albicans*, and usually requires a predisposing factor, such as antibacterial therapy, diabetes, pregnancy  
10 or immunodeficiency. Mucocutaneous candidiasis is known as thrush. Chromoblastomycosis may be caused by opportunistic pathogens including *Phialophora compacta*, *P. pedrosoi*, *P. verrucosa*, *Cladosporium carrionii* and *Rhinocladiella aquaspersa* following skin trauma, particularly in tropical and subtropical climates.

15

Coccidioidomycosis (valley fever, desert fever, desert rheumatism) may be caused by inhalation of spores of *Coccidioides immitis* and occurs particularly in arid and semi-desert areas of North, Central and South America. Cryptococcosis is caused by the fungus *Cryptococcus neoformans*. It is rare in normal  
20 individuals, but important in immunocompromised patients, often occurring as cryptococcal meningitis.

Endocarditis may be caused by fungi such as *Aspergillus* or *Candida*, as may eye infections. *Cephalosporium*, *Fusarium*, *Blastomyces*, *Cryptococcus* and  
25 *Sporothrix* may also cause eye infections.

Histoplasmosis may be caused by *Histoplasma capsulatum*, found in soil and in bird and bat excrement. It is a systemic infection that is endemic in central USA and central Africa (mainly *H. capsulatum* var *duboisii*).

Mucormycosis is caused by *Rhizopus* or *Rhizomucor* species and usually affects only immunocompromised individuals. Mycetoma is a tropical and subtropical disease caused by *Madurella mycetomatis*, *M. grisea* or *Pseudallescheria boydii* which enter the tissue through skin trauma. Paracoccidioidomycosis is a disease principally of Central and South America caused by *Paracoccidioides brasiliensis*.

Pneumocystis carinii pneumonia is caused by *Pneumocystis carinii*, which is thought to be a fungus. It is of particular significance in immunocompromised patients, particularly those with AIDS.

Skin infections include the dermatophytoses, pityriasis versicolor and candidiasis (discussed above), as well as forms of other fungal infections discussed above, such as aspergillosis. Dermatophytoses include ringworm and tinea (including athlete's foot), and are caused by *Epidermophyton*, *Microsporum* and *Trichophyton* spp. Pityriasis versicolor (tinea versicolor) is caused by *Malassezia furfur* and is more common in tropical than temperate climates and exposure to the sun may trigger the infection.

Sporotrichosis is caused by *Sporothrix schenckii* and is seen mainly in the Americas and Africa. It occurs in cutaneous and extracutaneous forms.

Further information regarding fungal infections for which the compounds of the invention may be useful may be found in, for example, the *Martindale Pharmacopoeia*, 32<sup>nd</sup> edition.

It will be appreciated that fungal infections may be more serious in immunocompromised hosts, for example a human with AIDS (acquired

immunodeficiency syndrome); thus, yeast/fungi that are of low pathogenicity in otherwise healthy individuals may be capable of causing more severe disease in an immunocompromised individual.

## 5 Kit of parts

A further aspect of the invention is a kit of parts comprising means useful for carrying out a screening method of the invention.

10 Thus, a kit of parts of the invention may comprise a first host yeast cell wherein the yeast cell is (a) not capable of expressing the said yeast cell protein kinase or kinases and (b) is capable of expressing the said equivalent protein kinase derivable from a first source

and

15 2) a second host yeast cell wherein the yeast cell is (a) not capable of expressing the said yeast cell protein kinase or kinases and (b) is capable of expressing the said equivalent protein kinase derivable from an source other than the first source.

## 20 Figure Legends.

**Figure 1.** Comparison of the primary structures of Pkh1 and Pkh2 with PDK1.

(a) Alignment of the deduced amino acid sequences of yeast Pkh1 and Pkh2 with the catalytic domains of human PDK1 and its *Drosophila* homologue, DSTPK61 [27], carried out using the CLUSTAL W program. Identical residues are denoted  
25 by white-on-black letters, and similar residues by grey boxes. In our work, five independent *PKH1* clones generated by PCR all differed from the sequence deposited in the *Saccharomyces* Genome Database, and indicated that Phe187 (TTC) should actually be Ile (ATC). (b) Schematic diagrams of the structures of



PDK1-related proteins. Blue boxes indicate the catalytic domain in each protein kinase and green boxes indicate PH domains.

**Figure 2.** Deletion of both *PKH1* and *PKH2* produces non-viable cells.

- 5 (a) Growth of spores from a representative tetratype tetrad from strain AC306 (*MATa/MATα PKH1/pkh1Δ::HIS3 PKH2/pkh2Δ::TRP1*). The deduced *pkh1Δ* *pkh2Δ* spore is inviable. (b) Verification by PCR using the appropriate primers indicated the presence of the expected deletion-substitution alleles. Presence of the *HIS3* and *TRP1* markers inserted into *PKH1* and *PKH2*, respectively, are  
10 denoted by a shift of the size of the resulting product from about 0.5 kbp in the intact loci, to 1.2 kbp (*pkh1Δ::HIS*) or 1.3 kbp (*pkh2Δ::TRP1*).

**Figure 3.** Genetic evidence that *PKH1* and *PKH2* are functional homologues.

- (a) Strain AC306 (*MATa/MATα PKH1/pkh1Δ::HIS3 PKH2/pkh2Δ::TRP1*),  
15 transformed with plasmid pYES2-*PKH1*, which is marked with *URA3*, was sporulated. Verification by PCR of the genotype of two derived haploids, *PKH1* *PKH2* [pYES2-*PKH1*] (spore A) and *pkh1Δ* *pkh2Δ* [pYES2-*PKH1*] (spore B).  
(b) Four spores derived from a tetratype ascus of the diploid described in (a), all carrying pYES2-*PKH1*, were plated either on medium lacking uracil (right) to  
20 select for the presence of the plasmid, or on medium containing 5-FOA (left) to select for loss of the plasmid [40]. In the absence of the plasmid-borne copy of *PKH1*, *pkh1Δ* *pkh2Δ* cells are unable to continue vegetative proliferation.

**Figure 4.** Intact human PDK1 or PDK1-ΔPH rescue the inviability of *pkh1Δ* *pkh2Δ* cells.  
25

- (a) Derivatives of strain AC306 (*MATa/MATα PKH1/pkh1Δ::HIS3 PKH2/pkh2Δ::TRP1*), transformed with either YEplac195-PDK1, YEplac195-PDK1-ΔPH, or YEplac195-*PKH1*, all marked with *URA3*, were sporulated.

Verification by PCR of the genotype of two derived double mutant spores maintained by expression of either authentic yeast *PKH1* (spore A) or human PDK1 (spore B). As expected, only the haploid carrying YEplac195-PDK1 possesses a PDK1-derived sequence. (b) Four spores derived from tetratype asci of the diploids described in (a), carrying either YEplac195-PDK1 or YEplac195-PDK1- $\Delta$ PH, as indicated, were plated either on medium lacking uracil (top left) to select for the presence of the plasmids (only one representative plate is shown), or on medium containing 5-FOA (top right and bottom right, respectively) to select for loss of the plasmids. The *pkh1* $\Delta$  *pkh2* $\Delta$  cells are able to grow only when the plasmids expressing human PDK1 or PDK1- $\Delta$ PH are present.

**Figure 5.** Both yeast Pkh1 and human PDK1 phosphorylate and activate human PKB in a PtdIns[3,4,5]P<sub>3</sub>- or PtdIns(3,4)P<sub>2</sub>-dependent manner.

Purified GST-PKB was incubated for 30 min at 30°C with either GST-Pkh1 (a) or GST-PDK1 (c) in the presence of 100  $\mu$ M ATP and phospholipid vesicles containing 100  $\mu$ M PtdCho, 100  $\mu$ M PtdSer, and the various PtdIns lipids indicated, all at a final concentration of 10  $\mu$ M. Reactions were terminated by adjusting the mixtures to a final concentration of 1% (by vol) Triton X-100 [17] to dissolve the lipid vesicles, and the resulting increase in specific activity (U/mg) of GST-PKB was then determined in the presence of [ $\gamma$ -<sup>32</sup>P]ATP, as described in Materials and Methods. The increase in specific activity given is that relative to control incubations in which GST-Pkh1 or GST-PDK1 were omitted. To measure the amount of incorporation into GST-PKB catalysed by either GST-Pkh1 (b) or GST-PDK1 (d), reactions were conducted in the presence of 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP and terminated by adjusting the solutions to a final concentration of 1% SDS. The resulting denatured samples were then subjected to SDS-PAGE. Extent of phosphorylation was assessed by autoradiography of the Coomassie blue-stained band corresponding to GST-PKB $\alpha$ . Abbreviations: SA-PI[3,4,5]P<sub>3</sub>

is *sn*-1-stearoyl, 2-arachidonyl D-PtdIns[3,4,5]P<sub>3</sub>; DP-PI[3,4,5]P<sub>3</sub> is *sn*-1,2-dipalmitoyl D-PtdIns [3,4,5]P<sub>3</sub>; DP-PI[3,4]P<sub>2</sub> is *sn*-1,2-dipalmitoyl D-PtdIns[3,4]P<sub>2</sub>; DP-PI[3]P is *sn*-1,2-dipalmitoyl D-PtdIns-3P. PI[4,5]P<sub>2</sub> was purified from brain extract.

5

**Figure 6.** Consensus sites for PDK1- and PDK2-dependent phosphorylation, and comparison of the primary structures of Ypk1 and Ykr2 with their closest human homologues.

(a) Conserved motifs predicted to be phosphorylated by PDK1-like and PDK2-like enzymes are indicated with the residue phosphorylated shown in boldface type. In all cases, the putative PDK2 site is located 157-166 residues C-terminal to the PDK1 site. GenBank accession numbers: PKB $\alpha$ : X65687; p70S6K $\alpha$ : M60725; SGK: Y10032; PKC $\zeta$ : L07032; Ypk1: P12688; Ykr2: P18961; Pkc1: M32491; and Sch9: U00029. (b) In the alignment of Ypk1 and Ykr2 to each other and to mammalian SGK, PKB $\alpha$ , p70 S6 kinase and  $\beta$ ARK, identities to Ypk1 are indicated by white-on-black letters.

15

**Figure 7.** Expression of either Ypk1 or Ykr2 is required for viability.

Strain YES7 (*MATa/MAT $\alpha$  YPK1/ypk1 $\Delta$ ::HIS3 YKR2/ykr2 $\Delta$ ::TRP1*), transformed with the *LEU2*-marked plasmid, p*GAL-YKR2*, were sporulated under conditions (galactose-containing media) that induce expression of *YKR2* from the plasmid. The four spores of a tetatype ascus derived from this diploid were recovered on medium lacking leucine to demand the presence of the plasmid. When subsequently streaked out on the same medium containing glucose as the carbon source, which represses expression of the plasmid-borne *YKR2* gene, the *ypk1 $\Delta$  ykr2 $\Delta$*  double mutant cells are unable to continue vegetative proliferation. In contrast, the cells expressing either *YPK1* or *YKR2* (or both) from their normal chromosomal loci remain viable.

20

25

**Figure 8.** SGK, a PKB-related enzyme, rescues the inviability of *ypk1Δ ykr2Δ* cells.

(a) Strain YPT28 (*MATa ypk1Δ ykr2Δ*) carrying pYKR2, a *URA3*-marked plasmid that expresses *YKR2* from its authentic promoter, was generated as described in

5 Materials and methods and then transformed with either empty *LEU2*-marked vectors (pAD4M or YEp351GAL) or the same vectors expressing from either the *ADHI* or the *GALI* promoters, as indicated, either *YPK1*, *YKR2*, SGK, PKB $\alpha$ , p70 S6 kinase or  $\beta$ ARK. When streaked onto medium selective for the presence of both plasmids (left), all of the transformants grew well. However, when  
10 plated on the same medium containing 5-FOA (right), to demand loss of the *URA3*-marked plasmid expressing *YKR2*, only cells expressing SGK were able to sustain normal growth of the *ypk1Δ ypk1Δ* cells, and did so as efficiently as authentic *YPK1* or *YKR2*. Survival of a few colonies was reproducibly observed for cells expressing PKB $\alpha$ , suggesting that only very high levels of PKB $\alpha$   
15 expression can support the continued growth of Ypk1- and Ykr2-deficient cells.

(b) The temperature-conditional strain YPT40 (*MATa ypk1-1<sup>ts</sup> ykr2Δ*) was constructed as described in Materials and methods and then transformed with the indicated plasmids, described in (a). The transformants isolated at permissive temperature (left) were then tested for their ability to continue to grow at the  
20 restrictive temperature (right). Again, SGK was able to substitute for Ypk1 and Ykr2 function and permit growth just as well as *YPK1* itself. Weak complementation by PKB was again observed, in that microcolonies indicative of very slow growth were reproducibly recovered at the non-permissive temperature.

25

**Figure 9.** Pkh1 phosphorylates and activates yeast Ypk1 and human SGK in a 3-phospho-inositide-independent manner.

Either GST-Ypk1 (a) or GST-SGK (c) were incubated for 30 min at 30°C with GST-Pkh1 or GST-PDK1, as indicated, in the presence of 100  $\mu$ M ATP, with

and without phospholipid vesicles containing 100  $\mu$ M PtdCho, 100  $\mu$ M PtdSer and 10  $\mu$ M of the D-enantiomer of *sn*-1-stearoyl, 2-arachidonyl D-PtdIns[3,4,5]P<sub>3</sub> ("PIP3"). Reactions were terminated and the degree of activation assessed as indicated in the legend to Fig. 5. To measure the amount of incorporation into GST-Ypk1 (b) or GST-SGK (d) catalysed by either GST-Pkh1 or GST-PDK1, as indicated, reactions were carried out in the presence of 100 M [ $\gamma$ -<sup>32</sup>P]ATP and the products were analysed by SDS-PAGE followed by autoradiography, as described in the legend to Fig. 5.

10 **Figure 10.** Phosphorylation of Thr504 in Ypk1 is essential for its activation by Pkh1.

(a) GST-Ypk1, GST-Ypk1(T504D), GST-Ypk1(T662D) or GST-Ypk1(T504D T662D) were incubated with either GST-Pkh1 or a catalytically-inactive derivative, GST-Pkh1(KD), as indicated, for 30 min at 30°C with ATP (100  $\mu$ M). Reactions were terminated and the degree of activation assessed as indicated in the legend to Fig. 5. To follow the amount of incorporation into GST-Ypk1 and its various derivatives (b), reactions were carried out in the presence of 100  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP and the products were analysed by SDS-PAGE followed by autoradiography, as described in the legend to Fig. 5.

20

**Figure 11.** Parallels between PDK1-dependent signaling pathways in animal cells and Pkh1- and Pkh2-dependent signaling in yeast.

See text for further details.

25 **Figure 12.** Nucleotide sequence of rat SGK.

**Figure 13.** Amino acid sequence of rat SGK (gi|477098|pir|A48094 serum and glucocorticoid-regulated kinase - rat).

**Example 1: Functional homologues of mammalian 3-phosphoinositide-dependent protein kinase-1 (PDK1) and protein kinase B (PKB/c-Akt) are components of a signaling pathway in the yeast *Saccharomyces cerevisiae***

5

**Background:** In animal cells, recruitment and activation of phosphatidylinositol 3-kinase by the binding of growth factors to their cognate receptors generates 3-phosphoinositides. These lipids induce 3-phosphoinositide-dependent protein kinase-1 (PDK1) to phosphorylate and activate a variety of target protein kinases, including protein kinase B (PKB/c-Akt) and p70 S6 kinase, that subsequently mediate appropriate physiological responses.

**Results:** The *Saccharomyces cerevisiae* genome encodes two protein kinases (PKH1 and PKH2 gene products) whose catalytic domains are 72% identical to each other and 50% identical to human or *Drosophila* PDK1. Both *pkh1Δ* and *pkh2Δ* single mutants are viable, but a *pkh1Δ pkh2Δ* double mutant is inviable, indicating that Pkh1 and Pkh2 have essential, but overlapping, functions. Expression of human PDK1 permits growth of *pkh1Δ pkh2Δ* cells. The yeast genome also encodes two other protein kinases (YPK1 and YKR2 gene products) whose catalytic domains are 88% identical to each other and 58% identical to serum- and glucocorticoid-induced protein kinase (SGK), an enzyme closely related to PKB (56% identity). Either a *ypk1Δ* or a *ykr2Δ* single mutant is viable, but a *ypk1Δ ykr2Δ* double mutant is inviable, indicating that Ypk1 and Ykr2 also have essential, but overlapping, functions. Growth of *ypk1Δ ykr2Δ* cells is fully rescued by expression of rat SGK, weakly by mouse PKB, and not at all by rat p70 S6 kinase. Pkh1, expressed in 293 cells as a fusion to glutathione S-transferase (GST), phosphorylates and activates mammalian PKB and SGK. Likewise, *in vitro* Pkh1 activates Ypk1 by phosphorylating the residue

(T504) in the kinase domain equivalent to those in PKB and SGK that are phosphorylated by PDK1. Activation of Ypk1 by Pkh1 does not require phosphatidylinositol-3,4,5-*tris*phosphate (PtdIns[3,4,5]P<sub>3</sub>), consistent with the lack of pleckstrin homology (PH) domains in these yeast proteins, whereas  
5 activation of human PKB by Pkh1 is dependent on PtdIns[3,4,5]P<sub>3</sub>. The minimum consensus sequence for phosphorylation by Ypk1 is Arg-X-Arg-X-X-Ser-Hyd (where Hyd is a bulky hydrophobic residue), as found for PKB.

**Conclusions:** These results demonstrate that Pkh1 and Pkh2 are functional  
10 homologues of PDK1, that Ypk1 and Ykr2 are functional homologues of SGK (and perhaps PKB), and that, as in animal cells, these enzymes are components of a protein kinase cascade that is required for cell growth and viability.

### Background

15 Protein kinase B (PKB) [1], also called RAC (for “Related to PKA and PKC”) kinase [2], is the mammalian homologue of a retroviral oncogene product, v-Akt [3], and is, therefore, also designated c-Akt. There are several reasons for the current interest in this enzyme and its function. First, PKB is activated within minutes in response to insulin and other growth factors, and activation is  
20 prevented by inhibitors of phosphatidylinositol (PtdIns) 3-kinase [4-6]. Second, increasing evidence indicates that PKB mediates a number of the actions of insulin, including stimulation of glucose and amino acid uptake, glycogen and protein synthesis and, in cardiac muscle, glycolysis (reviewed in [7, 8]), as well as induction of the transcription of specific genes [9, 10]. Third, the PKB  
25 isoform is overexpressed in a significant percentage of ovarian and pancreatic cancers [11, 12], and the PKB isoform is elevated in some breast cancers [13]. Fourth, PKB action provides a survival signal that protects cells from apoptosis induced in a variety of ways [14, 15]. Hence, activation of PKB by gene amplification and other mechanisms may contribute to the generation of

malignant cells that are able to proliferate independently of extracellular growth and survival signals.

PKB phosphorylates proteins and peptides at serine or threonine residues that lie  
5 in an -Arg-X-Arg-X-X-Ser/Thr- motif [16]. In insulin signal transduction, two  
physiological substrates of PKB appear to be the protein kinase, glycogen  
synthase kinase-3 (GSK3) [17, 18], and the cardiac isoform of PFK2 [7, 19].  
Phosphorylation by PKB inhibits GSK3 activity, leading to dephosphorylation  
and activation of glycogen synthase and protein synthesis initiation factor eIF-2B  
10 [20]. These events presumably contribute to the insulin-induced stimulation of  
glycogen synthesis and protein synthesis, respectively. PKB activates cardiac  
PFK2, which seems to underlie the insulin-induced stimulation of glycolysis  
observed in the heart. In the protection of cells against apoptosis, BAD appears  
to be one of the physiological substrates of PKB [14, 15]. In its  
15 dephosphorylated form, BAD interacts with the Bcl family member, BclXL,  
thereby inducing apoptosis in some cells. However, when phosphorylated by  
PKB at Ser136, BAD dissociates from BclXL, interacts with 14-3-3 proteins  
instead, and apoptosis is prevented [21].

20 Activation of PKB in response to insulin or growth factors requires  
phosphorylation [22, 23], which occurs at two sites [23]. These are Thr308,  
located in a -Thr-Phe-Cys-Gly-Thr-X-Glu-Tyr- motif within the "activation loop"  
of the catalytic domain between conserved subdomains VII and VIII, and Ser473,  
situated in the hydrophobic motif -Phe-X-X-Phe-Ser-Phe-, close to the C-  
25 terminus. Both sequence motifs are present in a number of protein kinases that  
play important roles in signal transduction [7, 24, 25]. Phosphorylation of both  
sites in PKB is prevented by inhibitors of PtdIns 3-kinase [22]. Thr308 is  
phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) [26,



27] and Ser473 by a distinct protein kinase activity, termed PDK2 activity, which may be possessed by PDK1 (Balendran *et al* (1999) *Current Biology* 9, 393-404).

PKB can only be activated by PDK1 *in vitro* in the presence of lipid vesicles  
5 containing PtdIns[3,4,5]P<sub>3</sub> or PtdIns[3,4]P<sub>2</sub> [26]. The former lipid is generated from PtdIns[4,5]P<sub>2</sub> by PtdIns 3-kinase, and can be converted to the latter by one of several different classes of 5-phosphoinositide 5-phosphatases [28, 29]. The 3-phosphoinositides bind to a pleckstrin homology (PH) domain at the N-terminus of PKB [30, 31], presumably inducing thereby a conformational change that  
10 makes Thr308 accessible to PDK1. PDK1 also possesses a PH domain, C-terminal to the catalytic domain [27], that binds PtdIns[3,4,5]P<sub>3</sub> and PtdIns[3,4]P<sub>2</sub> even more tightly than does the PH domain of PKB [32, 33]. The interaction of PDK1 with 3-phosphoinositides enhances activation of PKB *in vitro*, probably by facilitating encounter of PDK1 and PKB via their mutual binding to lipid vesicles  
15 [33]. There is increasing evidence that, in addition to PKB, PDK1 activates a number of other protein kinases *in vivo*, such as p70 S6 kinase [25, 34], certain PKC isoforms [35] and, more recently, SGK [36], by phosphorylating a threonine residue that lie at an equivalent position and within the same sequence motif as Thr308 of PKB. Like PKB, these enzymes also contain the second,  
20 hydrophobic, phosphorylation consensus sequence, situated 160-165 residues C-terminal to the PDK1-dependent phosphorylation site [7] and, hence, are likely to be substrates for PDK2 protein kinase activity.

If enzymes of the PDK1 and PKB class perform functions vital to signaling in all  
25 eukaryotic cells, then these molecules should be evolutionarily conserved. We have shown previously that this pathway is apparently present in the fruit fly, *Drosophila melanogaster* [27]. Here we demonstrate both genetically *in vivo* and biochemically *in vitro* that PDK1-like and PKB-like protein kinases exist even in the unicellular eukaryotic microbe, *Saccharomyces cerevisiae* (baker's yeast).

Moreover, we show that these enzymes are indispensable for cell growth and viability. Additional findings suggests that, as in animal cells, the PDK1-like gene products are likely to play a role in activating other classes of protein kinases, in addition to the PKB-like enzymes.

5

## Results

### ***PKH1* and *PKH2* encode homologues of mammalian PDK1**

The genome of the yeast *Saccharomyces cerevisiae* contains two, previously uncharacterised open reading frames (YDR490c and YOL100w) that encode protein kinases whose catalytic domains share 50% amino acid sequence identity with either human or *Drosophila* PDK1 and are 72% identical to each other (Fig. 1A). Hence, these loci were designated *PKH1* and *PKH2*, respectively (for “PKB-activating Kinase Homologues 1 and 2”). *PKH1* is located on the right arm of chromosome IV [37] and encodes a 766-residue protein (86 kDa); *PKH2* is situated on the left arm of chromosome XV [38] and encodes a 1,081-residue protein (121 kDa). The Pkh1 and Pkh2 polypeptides also contain N-terminal extensions and long C-terminal extensions (Fig. 1B) and are much less similar to each another in these regions (27% identity). In these domains, there is no significant homology with the non-catalytic regions of PDK1 and no significant homology with other known proteins. In particular Pkh1 and Pkh2 lack the PH domain that is found near the C-termini of both the human and *Drosophila* PDK1.

### ***PKH1* and *PKH2* are essential genes that are functionally redundant**

As a means to determine if *PKH1* and *PKH2* are expressed genes, the phenotypic effect of loss-of-function mutations in these loci was examined. For this purpose, each open reading frame was deleted and replaced with a selectable marker. The resulting alleles, *pkh1Δ::TRP1* and *pkh2Δ::HIS3*, were used to replace the normal chromosomal loci by homologous recombination. Both a haploid *pkh1Δ::TRP1*

mutant (AC301) and a haploid *pkh2Δ::HIS3* mutant (AC303) grew normally and indistinguishably from congenic *PKH1+* and *PKH2+* haploids isolated from the same tetrad. In addition, the *pkh1Δ* and *pkh2Δ* single mutants displayed no apparent phenotype when the cells were challenged under various conditions, including high concentrations of salt or caffeine, different carbon sources, and different temperatures, or when subjected to heat shock.

To determine if *PKH1* and *PKH2* might share a common function, strain AC301 (*MAT pkh1Δ::TRP1*) was crossed with strain AC303 (*MATa pkh2Δ::HIS3*). Upon sporulation of the resulting doubly-heterozygous diploid (AC306), the majority of the 30 tetrads dissected, yielded three viable and one non-viable spore (Fig. 2A). The viable spores were analysed both by plating on appropriate selective media and by PCR (Fig 2B). None of the viable haploid cells were Trp<sup>+</sup> and His<sup>+</sup>, and none carried both the *pkh1Δ* and the *pkh2Δ* mutations. Microscopic observation of the non-viable spores revealed that most germinated and underwent two or three cycles of cell division before ceasing to grow. Hence, *pkh1Δ pkh2Δ* double mutants are inviable, indicating that *PKH1* and *PKH2* encode genes that are functionally redundant and that share some role that is essential for cell growth and survival.

20

To confirm that the lethality of *pkh1Δ pkh2Δ* cells was due solely to the absence of *PKH1* or *PKH2* function, AC306 was transformed with either YEplac195-*PKH1*, a *URA3*-marked plasmid expressing *PKH1* from its endogenous promoter, or the empty vector (YEplac195), and the resulting Ura<sup>+</sup> transformants were subjected to sporulation and tetrad dissection. It was possible to identify many Trp<sup>+</sup> His<sup>+</sup> and Ura<sup>+</sup> spore clones from the diploid transformed with YEplac195-*PKH1*, but not from that transformed with the empty vector (data not shown). Thus, the *pkh1Δ pkh2Δ* double mutant was able to survive if it expressed *PKH1* from a plasmid. Likewise, when AC306 was transformed with a *URA3*-marked

25

plasmid (pYES2) expressing either *PKH1* (Fig. 3A) or *PKH2* (data not shown) from the *GAL1* promoter, it was possible to obtain viable *pkh1Δ pkh2Δ* spores, even when the cells were propagated on glucose (presumably because the particular constructs used are not efficiently repressed on this carbon source [39]). Moreover, when the *pkh1Δ pkh2Δ* cells harboring either pYES2-*PKH1* (Fig. 3B) or pYES2-*PKH2* (data not shown) were plated on medium containing 5-fluoro-orotic acid (5-FOA), which selects for cells that lack a functional *URA3* gene [40] and, hence, for loss of the *URA3*-marked pYES2 plasmid, the *pkh1Δ pkh2Δ* cells were no longer capable of growing (Fig 3B).

#### Human PDK1 is a functional homologue of Pkh1 and Pkh2

To determine if Pkh1 and Pkh2 are homologous in function, as well as in sequence, to PDK1, AC306 was transformed with YEplac195 expressing either full-length human PDK1 or human PDK1 lacking its C-terminal PH domain (PDK1-ΔPH), under control of the authentic *PKH1* promoter. Expression of either PDK or PDK1-ΔPH from this vector permitted the recovery of viable *pkh1Δ pkh2Δ* spores (Fig. 4B). Moreover, selection against these *URA3*-marked plasmids by plating on 5-FOA medium prevented the growth of the *pkh1Δ pkh2Δ* double mutants, but not a *pkh1Δ* or a *pkh2Δ* single mutant carrying the same plasmids (Fig. 4B). Viable *pkh1Δ pkh2Δ* double mutant spores could also be recovered when AC306 was transformed with a vector (pYES2) expressing either PDK1 or PDK1-ΔPH from the *GAL1* promoter, even when the cells were germinated and grown on glucose-containing medium, presumably because the particular constructs used are not efficiently repressed on this carbon source (data not shown). These results demonstrate that the catalytic domain of PDK1 is sufficient to substitute for the function of Pkh1 and Pkh2 and suggests that PDK1 is able to phosphorylate the same essential target substrates in yeast cells as Pkh1 and Pkh2.

### Purification and characterisation of Pkh1 kinase activity

The *PKH1* coding region was expressed, as a fusion to GST, in human 293 cells and purified from cell lysates by affinity chromatography on glutathione-  
5 Sepharose. The material obtained showed a single Coomassie blue-staining band on SDS-PAGE that migrated as a 112 kDa species, in agreement with the molecular mass expected for the GST-Pkh1 fusion. A catalytically-inactive (“kinase-dead”) mutant altered in a conserved residue known to be critical for binding the  $Mg^{2+}$ -ATP substrate, Pkh1(D267A), was also expressed and purified.  
10 This GST-KD-Pkh1 construct served as a control.

The activity of GST-Pkh1 was assessed *via* its ability to phosphorylate and activate GST-PKB, a known substrate of human PDK1. Phosphorylation of PKB was assessed by the incorporation of label in reactions containing  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ .  
15 Activity of PKB was measured *via* its ability, after its incubation with GST-Pkh1, to phosphorylate a specific peptide substrate, Crosstide [17]. Yeast Pkh1 was able to activate human PKB (Fig. 5A), provided that lipid vesicles containing 3-phosphoinositides that are known to interact with the PH domain of PKB were present. There was an excellent correlation between the Pkh1-  
20 dependent phosphorylation of PKB (Fig. 5B) and the degree of activation observed (Fig. 5A). No activation or phosphorylation of PKB was observed if PtdIns[3,4,5] $P_3$  or PtdIns[3,4] $P_2$  were omitted, or replaced by PtdIns[4,5] $P_2$  or PtdIns-3P. As observed with human PDK1 (Fig. 5C and 5D), Pkh1 phosphorylated and activated PKB more efficiently in the presence of the  
25 naturally-occurring stearyl-arachidonyl derivative of PtdIns[3,4,5] $P_3$  than in the presence of the dipalmitoyl derivative. GST-KD-Pkh1 did not activate or phosphorylate PKB under any condition tested (data not shown).

To determine what residue in PKB was phosphorylated by Pkh1, GST-PKB was phosphorylated to completion by prolonged incubation in the presence of [ $\gamma$ - $^{32}$ P]ATP, cleaved with trypsin, and the resulting digest resolved by HPLC on an RP-C<sub>18</sub> column. Only one major labelled phosphopeptide was observed (data not shown). The elution position of this species was congruent with that of the phosphopeptide (residues 308-328) obtained by tryptic digestion of PKB phosphorylated by human PDK1. No  $^{32}$ P-labelled material eluted at the position corresponding to the PKB peptide that contains Ser473. The peptide labelled by Pkh1 contained phosphothreonine (and no other phosphoamino acid) and all the radioactivity was released after one cycle of Edman degradation (data not shown). This analysis establishes that yeast Pkh1 phosphorylates PKB at Thr308, the same residue phosphorylated by human PDK1. Hence, the yeast and human enzymes display the same specificity *in vitro* using this substrate.

#### 15 ***YPK1* and *YKR2* encode homologues of mammalian PKB**

PKB is one of the founding members of the so-called AGC sub-family of protein kinases. Moreover, PDK1 is known to phosphorylate PKB and several other members of this sub-family at a specific motif that lies in the so-called "activation loop" situated between conserved elements VII and VIII in the catalytic domains of these enzymes (Fig. 6A). In addition, all of these targets also share a different consensus sequence at a C-terminal phosphorylation site for another protein kinase activity (PDK2). The *S. cerevisiae* genome encodes four, previously characterised protein kinases that possess both of these same motifs (Fig. 6A). The *YPK1* gene was originally identified by screening a yeast genomic library using a cDNA encoding the catalytic subunit of mammalian cyclic-dependent protein kinase as the probe [41]. Similarly, the highly-related *YKR2* gene, also called *YPK2* [42], was first identified by library screening with a mammalian PKC cDNA probe [43]. The *SCH9* gene was identified by its ability, when overexpressed, to rescue the lethality of cells defective in the activation of Ras

[44]. Finally, the *PKC1* gene was identified as a bona fide yeast homologue of major mammalian PKC isotypes [45]. However, among these four gene products, the catalytic domains of Ypk1 and Ykr2 share 88% identity, as well as extensive similarities across their N- and C-terminal extensions. Most significantly, among all other protein kinases in available databases, the catalytic domains of Ypk1 and Ykr2 share greatest similarity to members of the AGC subfamily (Fig. 6B), including mammalian SGK (58% identity) [36, 46], PKB (54% identity) and p70 S6 kinase (52% identity). ARK (50% identity) [47] is another AGC subfamily member but does not have consensus phosphorylation PDK1 motifs. This sequence homology raised the possibility that Ypk1 and Ykr2 may be physiological substrates for Pkh1 and Pkh2.

#### ***YPK1* and *YKR2* are essential genes that are functionally redundant**

It has been reported previously that cells lacking either Ypk1 or Ykr2 are viable, whereas cells lacking both Ypk1 and Ykr2 are inviable [42]. To confirm this observation, and to generate a strain in which the ability of potential mammalian homologues to rescue this inviability could be readily tested, otherwise isogenic *ypk1Δ::HIS3* and *ykr2Δ::TRP1* haploid strains (YES5 and YES1, respectively) were constructed and crossed to form a doubly heterozygous diploid (YES7). This diploid was transformed with a *LEU2*-marked plasmid expressing the *YKR2* gene under the tight control of the *GAL1* promoter, and a Leu<sup>+</sup> transformant was subjected to sporulation and tetrad dissection on galactose-containing medium. Under these conditions, most of the tetrads yielded four viable spores, and His<sup>+</sup> Trp<sup>+</sup> Leu<sup>+</sup> isolates were readily recovered; whereas, when the Leu<sup>+</sup> transformant was sporulated on glucose, no His<sup>+</sup> Trp<sup>+</sup> Leu<sup>+</sup> spores could be recovered (data not shown). Moreover, as expected if the absence of both *YPK1* and *YKR2* function is lethal, when the *ypk1Δ ykr2Δ* double mutant carrying *pGAL-YKR2*, previously maintained on galactose medium, was streaked on glucose medium, the cells failed to grow, whereas otherwise isogenic wild-type

cells or *ypk1Δ* and *ykr2Δ* single mutants carrying the same plasmid grew well on glucose (Fig. 7). These results demonstrate that *YPK1* and *YKR2* encode genes that are functionally redundant and that share some role that is essential for cell growth and survival. In our hands, however, a *ypk1Δ* mutant grows detectably  
5 more slowly than otherwise isogenic wild-type cells or a *ykr2Δ* mutant, either in liquid culture or on solid agar medium (data not shown).

**PKB-related enzyme, SGK, is a functional homologue of Ypk1 and Ykr2**

To test the ability of mammalian protein kinases to rescue the lethality of the  
10 *ypk1Δ ykr2Δ* double mutant, diploid YES7 was transformed with a low-copy-number (*CEN*) plasmid marked with *URA3* and expressing *YKR2* from its endogenous promoter. A Ura<sup>+</sup> transformant was sporulated, dissected, and a His<sup>+</sup> Trp<sup>+</sup> Ura<sup>+</sup> spore, representing a *ypk1Δ ykr2Δ* haploid maintained by expression of *YKR2* from the plasmid (strain YPT28), was recovered. YPT28  
15 was then transformed with either empty *LEU2*-marked, high-copy-number (2 μm DNA) vectors (pAD4M or YEp351GAL) or the same vectors expressing (from either the *GAL1* promoter or the constitutive *ADH1* promoter [48]) *YPK1*, *YKR2*, or cDNAs encoding rat SGK, mouse PKB/c-AKT, rat p70 S6 kinase, or bovine βARK. The nucleotide and amino acid sequences of rat SGK are shown in  
20 Figures 12 and 13, respectively. All of these strains are able to grow on galactose medium due to the presence of the plasmid expressing *YKR2*, which also demonstrated that expression of none of the heterologous protein kinases tested was deleterious to yeast cell growth (Fig. 8A). In contrast, when plated on the same medium containing 5-FOA, thereby demanding loss of the  
25 pYKR2(*URA3*) plasmid, the *ypk1Δ ykr2Δ* cells carrying the empty *LEU2*-marked vectors were unable to grow, whereas those harboring plasmids expressing either *YPK1* or *YKR2* remained viable, as expected. Likewise, any other protein kinase that is able to perform the essential function of Ypk1 and Ykr2 should also permit growth on 5-FOA. Of the four mammalian cDNAs tested, only SGK displayed



efficient complementation of the *ypk1Δ ypk1Δ* double mutant (Fig. 8A). However, we also reproducibly observed weak complementation by PKB, in that a small percentage of the colonies were able to survive (presumably representing cells expressing exceedingly high levels of PKB resulting from accumulation of the 2  $\mu$ m DNA plasmid due to its notoriously poor segregation efficiency [49]). All of the mammalian protein kinases were produced at high levels in yeast (grown on SCGal-Leu), as judged by immunoblotting with appropriate antibodies, and were active, as judged by assaying extracts of the yeast cells with appropriate specific substrates (data not shown); hence, the failure of p70 S6 kinase and  $\beta$ ARK to complement was not due to their lack of expression. Because p70 S6 kinase, in particular, is under such complex regulation in animal cells [50], truncations of the N-, C-, and both N- and C-termini [51] were also tested in the same way; although all were expressed, none was able to rescue the lethality of the *ypk1Δ ykr2Δ* cells (data not shown).

To confirm these results by an independent method for assessing the ability of the mammalian protein kinases to complement, the same plasmids described above were introduced into a yeast strain (YPT40) that displays temperature-conditional growth because it carries a null mutation (*ykr2Δ*) in *YKR2* and a temperature-sensitive (ts) mutation (*ypk1-1<sup>ts</sup>*) in *YPK1*, the latter of which was generated as described in detail in Materials and Methods. As expected, all of the transformants were able to grow at the permissive temperature (26°C), whereas the strain carrying an empty vector (YEp351GAL) was unable to survive at the restrictive temperature (35°C), but the strain expressing *YPK1* from the same vector grew well (Fig. 8B). As observed before, the cells expressing SGK also were able to grow well at 35°C, and the cells expressing PKB were able to grow weakly, in that microcolonies were observed outside of the heavy initial streak, whereas cells expressing p70 S6 kinase or  $\beta$ ARK did not grow (Fig. 8B).

**Ypk1 and mammalian SGK are efficient substrates for Pkh1**

Based on the observations described in the preceding sections, Ypk1 (and/or Ykr2) should be physiological substrates of Pkh1 (and/or Pkh2). In addition, yeast Pkh1 (and/or Pkh2) should be able to phosphorylate and activate mammalian SGK *in vitro*, as it has previously been shown that PDK1 is capable of phosphorylating and activating SGK [36]. To test these hypotheses, Ypk1 and SGK lacking the N-Terminal 60 amino acids [36] were expressed as GST fusion proteins in 293 cells and purified. Each purified protein yielded a single Coomassie blue-stained band on SDS-PAGE with an apparent mobility in good agreement with its expected molecular mass (data not shown). In the absence of any other factor, purified GST-Ypk1 displayed no detectable activity toward the peptide substrate (Crosstide); however, after pre-incubation with purified GST-Pkh1, GST-Ypk1 was activated and catalysed a readily detectable level of incorporation into the substrate in the presence or absence of lipid vesicles containing PtdIns[3,4,5]P<sub>3</sub> (Fig. 9A). Consistent with activation resulting from Pkh1-dependent phosphorylation, when [ $\gamma$ -<sup>32</sup>P]ATP was included in the reaction, incorporation of label into GST-Ypk1 was readily detected (Fig. 9B) and was present exclusively as phosphothreonine (data not shown). A "kinase-dead" derivative, GST-KD-Ypk1(D488A) (see Materials and methods), was phosphorylated by GST-Pkh1 but, as expected, was not catalytically-active (data not shown). Also, as predicted, GST-SGK was both phosphorylated and activated upon incubation *in vitro* with GST-Pkh1, even more efficiently than by GST-PDK1 (Fig. 9C and 9D).

As one means to map the Pkh1 phosphorylation site in Ypk1, a GST-Ypk1(T504D) mutant was expressed in 293 cells and purified. The altered residue is equivalent to the PDK1 target residue (Thr308) in PKB $\alpha$ . As expected, GST-Ypk1(T504D) was not detectably phosphorylated by Pkh1 (Fig. 10), consistent with the conclusion that Pkh1 phosphorylates Ypk1 at this residue.

Since GST-Ypk1(T504D) was neither constitutively active nor activated by Pkh1, Asp at this position cannot substitute for phosphothreonine to activate this protein kinase. Ypk1 also contains a putative consensus site (Thr662) for PDK2 phosphorylation (Fig. 6A), equivalent to Ser473 in PKB $\alpha$ . GST-Ypk1(T662D) expressed and purified from 293 cells was also inactive; however, this protein was phosphorylated and activated by Pkh1 in a manner identical to GST-Ypk1 itself (Fig. 10). A form of Ypk1 in which both Thr504 and Thr662 were mutated to Asp was also generated. This GST-Ypk1(T504D T662D) mutant also displayed no detectable activity, before or after incubation with Mg<sup>2+</sup>-ATP and GST-Pkh1, demonstrating that, unlike PKB $\alpha$ , mutation of these residues to Asp cannot substitute for phosphothreonine to produce a constitutively-active enzyme.

#### Substrate specificity of Ypk1 is similar to PKB

PKB $\alpha$  phosphorylates its substrates at the minimal consensus sequence Arg-X-Arg-X-X-Ser-Hyd, where Hyd is a bulky hydrophobic residue [16]. Using several series of peptide substrates of related sequence, efficiency of phosphorylation by Pkh1-activated Ypk1 was observed to be quite similar to that observed for PDK1-activated PKB (Table 2). Moreover, the effect of alterations on the rate of phosphorylation showed nearly identical trends. For example, as seen for PKB $\alpha$ , changing either Arg to Lys, or absence of the P-5 Arg, or absence of the P+1 hydrophobic residue, all drastically reduced or abolished phosphorylation by Ypk1.

#### Discussion

Here we have shown that *S. cerevisiae* contains protein kinases that are homologues of mammalian PDK1 and PKB family members, in terms of sequence, physiological function in vivo, and biochemical specificity in vitro. Specifically, we demonstrated, first, that two previously uncharacterised open reading frames, now designated the *PKH1* and *PKH2* genes, encode PDK1-like

protein kinases. The function(s) of Pkh1 and Pkh2 appear to overlap because loss of either enzyme produces no obvious phenotype, whereas a cell deficient in both enzymes is inviable. Expression of the N-terminal catalytic domain of mammalian PDK1 alone is sufficient to overcome the lethality of *pkh1Δ pkh2Δ* cells. Consistent with this finding, purified Pkh1 phosphorylates and activates known substrates of mammalian PDK1, including PKB and SGK and, where analysed, phosphorylates the same residue (Thr308 in PKB $\alpha$ ) as PDK1.

Next, we demonstrated that the *YPK1* and *YKR2* gene products are PKB-like protein kinases. In agreement with a previous report [42], we found that loss of Ykr2 produces no discernable phenotype, that absence of Ypk1 results in slower cell growth, and that a *ypk1Δ ykr2Δ* double mutant is inviable, indicating that these protein kinases also have some functional redundancy. Expression of SGK, a close relative of PKB $\alpha$ , that lacks an obvious N-terminal PH domain, rescues efficiently the inviability of *ypk1Δ ykr2Δ* cells. We observed that PKB itself was able to sustain only weakly the growth of the *ypk1Δ ykr2Δ* double mutant. This latter result is consistent with the prediction that PKB will exist in yeast largely in the inactive state because *Saccharomyces cerevisiae* lacks any enzymic machinery capable of generating either PtdIns[3,4,5]P $_3$  or PtdIns[3,4]P $_2$  [52-54]. Binding of these phospholipids is apparently necessary to relieve conformational constraints in PKB and thereby allow it to be an efficient substrate either for mammalian PDK1 or, as we have shown, for yeast Pkh1. This situation probably also explains why all four of the yeast proteins (Pkh1, Pkh2, Ypk1 and Ykr2) lack discernible PH domains and why Pkh1-dependent activation of Ypk1 is not influenced by the presence or absence of such 3-phosphoinositides. Indeed, Pkh1 does not bind PtdIns[3,4,5]P $_3$  under conditions where this phospholipid binds tightly to PDK1 (results not shown). Therefore, the fact that Pkh1-mediated activation of PKB only occurs efficiently in the presence of lipid vesicles containing PtdIns[3,4,5]P $_3$  or PtdIns[3,4]P $_2$  confirms unequivocally that these 3-

phosphoinositides exert their effects solely by interacting with the PH domain of PKB $\alpha$ . The signals that lead to activation of Pkh1 and Pkh2, or mobilize recruitment of these enzymes to particular subcellular locations, is not yet known. Likewise, the signals that promote encounter of Ypk1 and Ykr2 with Pkh1 and Pkh2 are also unknown.

Despite the fact that the catalytic domains of Ypk1 and Ykr2 are also rather similar to other AGC sub-family protein kinases from animal cells, including p70 S6 kinase and  $\beta$ ARK, these enzymes, although expressed in active form in yeast, were unable to support any detectable growth of cells deficient in Ypk1 and Ykr2. In addition to functional complementation in vivo, other results also indicate that Ypk1 and Ykr2 represent PKB-like enzymes. First, like its ability to phosphorylate and activate PKB and SGK, yeast Pkh1 is able to phosphorylate and activate Ypk1 in vitro. Second, mutagenesis studies indicated that Pkh1 phosphorylates Ypk1 at a residue (Thr504) that is equivalent to the position in PKB (Thr308) that is phosphorylated by PDK1 and by Pkh1. Finally, using a series of synthetic peptide substrates, we found that Pkh1-activated Ypk1 has a substrate specificity in vitro nearly identical to that displayed by PDK1-activated PKB [16].

20

Specifically, Ypk1 was able to phosphorylate Ser and Thr residues that lie in an Arg-X-Arg-X-X-Ser/Thr-Hyd motif. Thus, some of the physiological substrates of Ypk1 and Ykr2 may contain this consensus sequence. However, the yeast homologues of some of the known substrates of mammalian PKB $\alpha$  do not contain any perfect matches to this canonical site. For example, none of the four GSK3 homologues encoded in the *S. cerevisiae* genome [55] possess the Arg-X-Arg-X-X-Ser motif that is present at the N-terminus of the GSK3 isoforms targeted by mammalian PKBs. Also, yeast PFK2 lacks the two-C-terminal Arg-

25

X-Arg-X-X-Ser motifs found in the cardiac isoform of PFK2. Hence, the physiologically relevant substrates of Ypk1 and Ykr2 remain to be determined.

Taken together, these results indicate that Ypk1 (and, most likely, Ykr2) lie  
5 down-stream of Pkh1 (and/or Pkh2) in a protein kinase cascade that is essential  
for both the growth and survival of yeast cells. In mammalian cells, PDK1  
appears to be responsible for the activation of a number of different protein  
kinases of the AGC sub-family, in addition to PKB, including p70 S6 kinase,  
certain PKC isotypes and, most recently SGK [36], in agreement with results  
10 presented here. Are Ypk1 and Ykr2 the only substrates of Pkh1 and Pkh2 that  
are essential for yeast cell viability? Several observations indicate that, as for  
PDK1 in animal cells, Pkh1 and Pkh2 have multiple targets. First, human PKB  
can be partially activated by a T308D mutation (in the PDK1 site) or by a S473D  
mutation (in the PDK2 site), and almost fully activated by the simultaneous  
15 presence of both mutations [22]; nonetheless, expression of such a PKB (T308D  
S473D) mutant is unable to support vegetative growth of a *pkh1Δ pkh2Δ* double  
mutant (although, following germination of a *pkh1Δ pkh2Δ* spore, expression of  
such a construct does allow many more cycles of cell division before cessation of  
growth ensues than expression of PKB itself, yielding a microcolony visible  
20 under the microscope(results not shown)). Second, if Ypk1 or Ykr2 are the sole  
essential targets of Pkh1 and Pkh2, and possess any basal activity in the absence  
of Pkh1- and/or Pkh2-dependent phosphorylation, then rampant overexpression  
of these enzymes might bypass the need for Pkh1 and Pkh2 function; however,  
gross overproduction (approximately 50-fold) of either Ypk1 or Ypk2 from the  
25 strong *GAL1* promoter on multi-copy plasmids also does not rescue the inviability  
of *pkh1Δ pkh2Δ* cells (results not shown). In contrast to mammalian PKB $\alpha$ , it  
has not yet proved possible to generate constitutively-active variants of yeast  
Ypk1 or human SGK [36] by mutating to Asp the residues equivalent to Thr308  
and Ser473 in PKB $\alpha$ . Hence, we have been unable to test whether constitutively-

active forms of Ypk1 (or SGK) would be able to rescue the lethality of a *pkh1Δ* *pkh2Δ* double mutant. Third, one other potential Pkh1 and Pkh2 substrate, Pkc1, is known to be essential for yeast cell viability under standard growth conditions [45] because it sits at the head of a protein kinase cascade that controls a MAP kinase (Slt2/Mpk1) required for proper cell wall biosynthesis and survival under hypertonic stress (reviewed in [56]). However, again as expected if Pkh1 and Pkh2 have multiple essential targets, attempts to rescue the lethality of *pkh1Δ* *pkh2Δ* cells by overexpression of *PKC1* were also unsuccessful (results not shown). Yet another potential Pkh1 and Pkh2 target, Sch9, is thought, on the basis of genetic results, to serve as an effector enzyme in a pathway that is parallel to, and largely redundant in function with, the three yeast cyclic-AMP-dependent protein kinase catalytic subunits [44]. Indeed, additional genetic results suggest that activation of the  $\alpha$  subunit of a heterotrimeric G protein, Gpa2, but not activation of Ras2, leads to activation of Sch9 [57]. Like the absence of Ypk1 alone, loss of Sch9 causes slow growth, apparently due to prolongation of the G1 phase of the cell cycle [44].

Intriguingly, like mammalian PKB $\alpha$ , p70 S6 kinase, and SGK, the known and suspected substrates for yeast Pkh1 and Pkh2, including Ypk1, Ykr2, Pkc1 and Sch9, all also contain the consensus sequence (Phe-X-X-Phe/Tyr-Ser/Thr-Phe/Tyr) for phosphorylation by PDK2 protein kinase activity and at the same position (160-165 residues C-terminal) relative to the Thr known (or suspected) to be phosphorylated by Pkh1 (and, presumably, Pkh2). This conservation strongly suggests, first, that the *S. cerevisiae* genome encodes one (or more) protein kinases with a PDK2-like function and, second, that this enzyme(s) is likely to be or act coordinately with Pkh1 and/or Pkh2 to control activation of a number of target protein kinases, as observed for PDK1 and PDK2 activity in animal cells (Fig. 11).

## Conclusions

We have demonstrated here that the yeast, *S. cerevisiae*, possesses functional homologues of two effector enzymes, PDK1 and PKB, which are vital to the proper metabolic regulation and survival of mammalian cells in response to a variety of extracellular stimuli. This conservation suggests that the physiological roles of these two classes of protein kinase must be evolutionarily quite ancient and central to the growth and viability of all eukaryotic cells. The presence of these proteins in yeast should allow the application of genetic approaches to identify both upstream activators of the PDK1-like enzymes, Pkh1 and Pkh2, and downstream substrates for the PKB-like enzymes, Ypk1 and Ykr2. Such information may, in turn, shed light on previously unsuspected modes of regulation of mammalian PDK1 and previously unknown potential targets of PKB. In addition, analysis in yeast may assist in revealing the nature of the as yet uncharacterised PDK2 enzyme that is required, along with PDK1, for full activation of PKB in animal cells, and also presumably required for full activation of Ypk1 and Ykr2 in yeast.

## Materials and methods

### Cell culture

*S. cerevisiae* strains used in this study are described in Table 1, and were isogenic derivatives of either AYS927 (W303 background) or YPH499 (S288C background). Yeast cells were grown at 30°C in a rich medium (YPD) containing 1% yeast extract, 2% peptone (Difco) and 2% glucose, or in a synthetic minimal medium (S), containing either galactose (Gal) or glucose (Glc, or D, for dextrose) as the carbon source and supplemented with nutrients appropriate for maintaining selection for markers and plasmids [58]. Standard methods [58, 59] for genetic manipulation of yeast were used. *E. coli* cells (typically DH5 ) were grown at 37°C in Luria-Bertani medium [60] containing (when needed) 50 µg/ml ampicillin for plasmid selection.



*Recombinant DNA techniques*

Restriction enzyme digests, DNA ligations and other recombinant DNA procedures were performed as described [60]. Transformation of bacterial cells was achieved using electroporation. Transformation of yeast cells was accomplished by minor modifications of the lithium acetate method [61, 62]. The *PKH1* and *PKH2* genes used in this study, and the *YPK1* gene used in some constructions, were recovered from genomic DNA of strain AYS927 using PCR amplification with the Expand High Fidelity PCR System (Boehringer Mannheim), gel purified using the Kristal gelex kit (Cambridge Molecular Technologies, Cambridge, UK), and cloned first into the pCR2.1-TOPO vector using the TOPO system (Invitrogen). Site-directed mutagenesis was performed using the QuikChange Kit (Stratagene) following instructions provided by the manufacturer. All DNA constructs were verified by automatic DNA sequencing using an automated DNA Sequencer (Model 373; Applied Biosystems).

*PCR amplification of yeast genomic DNA*

The primers used to amplify the *PKH2* coding region were 5'-CG *GGA TCC GCC ACC ATG GAG CAG AAG CTG ATC TCT GAA GAG GAC TTG TAT TTG ATA AGG ATA ATT CCA TG*-3' (forward) and 5'-*ATA AGA AT GCG GCC GC TTA CGA CCT CTT CGA TTT TGC AG*-3' (reverse), and incorporated *Bam*HI and *Not*I sites, respectively (indicated by italics). The primers used to amplify the *PKH1* coding region were 5'-*ATA AGA AT GCG GCC GC TGC CAC C ATG GAG CAG AAC CTG TCT CTG AAG AGG ACT TG GGA AAT AGG TCT TGA CAG AGG*-3' (forward) and 5'-*ATA AGA AT GCG GCC GC TCA TTT TTC ATC TGT CCG TGT C*-3' (reverse), and incorporated *Not*I sites (indicated in italics). Both 5'-primers also contained a sequence encoding a 10-residue c-Myc epitope tag (underlined). The primers used to amplify the *YPK1* gene were: 5'-*GGA TCC GCC ACC ATG TAC CCA*

TAC GAT GTG CCA GAT TAC GCC TAT TCT TGG AAG TTT AAG-3'  
(forward) and 5'-*GGT ACC CTA TCT AAT GCT TCT ACC TTG C-3'*  
(reverse), and incorporated *Bam*HI and *Kpn*I restriction sites, respectively  
(italics). The initiator or termination codons in all these primers are also  
5 indicated (boldface type).

#### *Gene disruptions and strain constructions*

A PCR-based method [63] was used for disruption of the *PKH1* and *PKH2* genes.  
To generate the *pkh2Δ::HIS3* mutation, *HIS3* was amplified from pRS313 [64]  
10 using as primers 5'-AAG TAA CAT CTT GAT GAA CCG AGA AGC CAC  
TAA CTA GTT TT GTG CAC CAT AAT TTT CCG-3' (forward), where the  
underlined sequence corresponds to nucleotides -93 to 53 from the *PKH2*  
initiator codon and the remainder of the primer corresponds to nucleotides -326  
to -311 from the initiator codon of *HIS3*, and 5'-TAA GTA GCT TGA TGA  
15 AAA CAT TAG ATA AAA TTA CTA A TTA CCG TCG AGT TCA AGA G-  
3', where the underlined sequence corresponds to the nucleotides immediately  
after the *PKH2* stop codon (in boldface type) and the remainder corresponds to  
nucleotides 204 to 189 after the *HIS3* stop codon. The resulting 3.3 kb product  
was used for DNA-mediated transformation of a diploid strain (AYS927).  
20 Transformants were selected on SD-His plates, and disruption was verified by  
PCR analysis of from one of the His<sup>+</sup> isolates using appropriate primers. This  
heterozygous *PKH2/pkh2Δ::HIS3* diploid (AC200) was sporulated, and the  
resulting tetrads were dissected. Spore clones were analysed by plating on  
selective medium and confirmed by PCR to identify a haploid containing the  
25 *pkh2Δ::HIS3* disruption (AC303). To generate the *pkh1Δ::TRP1* mutation, the  
*TRP1* marker in pRS314 [64] was amplified using 5'-GCA CGT GTA CTT GCT  
TGA ATA CTG CTA CTA TAT CAT TAA T ATG GTA CTG AGA GTG  
CAC C-3' (forward), where the underlined sequence corresponds to nucleotides  
immediately upstream of the initiator codon (boldface type) and the remainder

corresponds to nucleotides situated -300 to 285 nucleotides from the initiator codon of *TRP1*, and 5'-TAT TAT GCA TTA CAC TTT CCC CTT CAC CAT GTC TTA CAT ATG CAT CCG CAG GCA AGT GCA C-3' (reverse), where the underlined nucleotides correspond to positions +69 to 25 after the *PKH1* stop codon and the remainder of the primer corresponds to the region situated +51 to 36 nucleotides after the *TRP1* stop codon. The resulting 2.4 kb product was used for transformation of AYS927, and transformants were selected on SD-Trp plates. Disruption was verified by PCR analysis from one of the Trp+ isolates using appropriate primers. This heterozygous diploid *PKH1/pkh1Δ::TRP1* (AC201) was sporulated, dissected, analysed, and a haploid spore containing the *pkh1Δ::TRP1* mutation (AC301) was identified.

In both the *ypk1-Δ1::HIS3* allele and the *ykr2-Δ1::TRP1* alleles [65], the coding sequences for the entire catalytic domains of both enzymes have been deleted and replaced with the indicated markers. To generate a *ypk1Δ ykr2Δ* double mutant, a *MATα ypk1Δ* strain (YES5) was mated to a *MATα ykr2Δ* strain (YES1) carrying *pYKR2(URA3)* (see below). The resulting diploid was subjected to sporulation and tetrad dissection, and a His+ Trp+ Ura+ spore, representing a *MATα ypk1Δ ykr2Δ* cell kept alive with the plasmid-borne *YKR2* gene, was designated YPT28. To create the temperature-conditional *ypk1-1<sup>ts</sup> ykr2Δ* strain (YPT40), a temperature-sensitive allele of *YPK1* was first generated, as follows. A genomic insert containing the *YPK1* gene [41], cloned into the *XbaI* site in the vector, pGEM3™ (Promega), and generously provided by Richard A. Maurer (then at the University of Iowa, Iowa City, IA), was excised as a 4.1 kb *XbaI-SalI* fragment and inserted into the *LEU2*-containing vector, pRS315 [64], yielding pRS315-*YPK1*. The sequence encoding the catalytic domain of *YPK1* was then amplified under moderately error-prone conditions using AmpliTaq™ DNA polymerase (Perkin Elmer Cetus), with pRS315-*YPK1* as the template and a 5'-primer (P1), 5'-TGC CCT CGA AGA CAT GGC-3', corresponding to a sequence beginning

at nucleotide 788 (where the first base of the ATG start codon is +1) and a 3'-primer (P2), 5'-CTT GAA CAC AGT AAG TAA CGG-3', corresponding to the flanking genomic sequence commencing 68-bp downstream of the stop codon. The resulting 1350-bp linear PCR product was gel purified and co-transformed into YPT28 along with a ~9 kb linear fragment of pRS315-*YPK1*, that had been generated by digestion with *Pst*I and *Nco*I, and gel purified. Transformants were selected on SCD-Leu plates at 26°C. This procedure allows for replacement of the corresponding sequence in the parent vector with potentially mutant sequences, and regeneration of circular plasmids, *via in vivo* repair of the gapped plasmid by recombination with homologous sequences present at each end of the PCR product [66, 67]. To determine which *LEU2*-containing plasmids expressed functional *YPK1* at 26°C, the Leu<sup>+</sup> transformants were subsequently replica-plated onto -Leu plates containing 5-fluoro-orotic acid (5-FOA) [39], which selects for loss of the *pYKR2(URA3)* plasmid initially present in the recipient *ypk1Δ ykr2Δ* strain. To determine which of the *YPK1*- and *LEU2*-containing plasmids harbored a temperature-sensitive allele of *YPK1*, the Leu<sup>+</sup> Ura<sup>-</sup> cells were tested by replica-plating for their ability to grow on SCD-Leu plates at 37°C. One transformant was identified that reproducibly failed to grow at this temperature. The *LEU2*-containing plasmid carried by this strain (*pypk1TS*) was recovered [68], and direct nucleotide sequence analysis of the *YPK1* open reading frame in the plasmid revealed the presence of two amino acid substitutions (I484T and Y536C). Sub-cloning and re-transformation confirmed that these mutations were sufficient to confer the temperature-sensitive (ts) phenotype, and this allele was designated *ypk1-I<sup>ts</sup>*. The *ypk1-I<sup>ts</sup>* allele was used to transplace the normal *YPK1* chromosomal locus in the *ykr2Δ* strain (YES1), as follows. First, PCR was used to generate a customized DNA fragment containing *Bam*HI and *Sma*I restriction sites 96 and 108 bp, respectively, downstream from the stop codon of the *YPK1* coding sequence in *pypk1TS* plasmid, and this fragment was substituted for the corresponding segment of the 3'-flanking region, yielding pINT. A 2.6

kb *ScaI*-*Bam*HI fragment containing the *HIS3* gene, excised from vector, pRS303 [64], was gel-purified and inserted into pINT that had been digested with *Bam*HI and *Sma*I, yielding pINT-HIS, which was able to confer both leucine and histidine prototrophy to a *leu2 his3* strain, YPH499 [64]. Finally, a 4.6 kb *Cla*I-*Xho*I fragment from pINT-HIS, containing the *ypk1-1<sup>ts</sup>* allele, the *HIS3* gene, and additional genomic DNA from the *YPK1* locus flanking the *HIS3* gene to its 3'-side, was gel purified and used for transformation of YES1. His<sup>+</sup> transformants were selected at 26°C, and then the presence of the integrated *ypk1-1<sup>ts</sup>* allele (and the absence of the normal *YPK1* locus) was confirmed by PCR analysis of DNA isolated from the transformants and by demonstrating that such cells were unable to grow at 37°C. One such isolate that met all of these criteria was designated strain YPT40.

### Plasmids

For expression of *PKH1*, *YPK1* as GST fusions in mammalian 293 cells, the corresponding coding sequences were excised from the appropriate pCR2.1-TOPO derivative and inserted into the mammalian expression vector, pEBG-2T [69]. PKB [26], PDK1 [27] and SGK lacking the N-terminal 60 residues [36] were subcloned into the pEBG-2T vector as described elsewhere. For expression of *PKH1* in yeast, a 2.1 kb fragment, from an internal *Sma*I site (situated about 250 bp downstream from the initiator codon) to an *Eco*RI site in the pCR2.1-TOPO vector, was first inserted into the 2 µm DNA vector, YEplac195 [70], that had been digested with *Sma*I and *Eco*RI, yielding YEplac195-2.1*PKH1*. To restore the 5'-end, a 1 kb fragment was amplified by PCR using the primers 5'-GCT TGA CTC AAT TAA GGC GAC-3' (forward), corresponding to nucleotides 628-634 upstream of the initiator codon, and 5'-ACA TGC TTA GTT AAC TCC-3' (reverse), corresponding to the region located 350 bp downstream of the initiator codon. The resulting product was first cloned into pCR2.1-TOPO, which was then digested with *Sma*I and *Sph*I to liberate a 0.9 kb fragment

that was inserted into the YEplac195-2.1*PKH1* construct that had been digested with *SphI* and *SmaI*, yielding YEplac195-*PKH1*, which contains the complete coding region of the *PKH1* gene plus 0.5 kb of its promoter region and carries the *URA3* gene as the selectable marker. To express *PKH1* under control of the  
5 *GAL1* promoter, a 2.3 kb *NotI-NotI* fragment containing the myc-tagged version of the entire *PKH1* coding sequence was inserted into the *URA3*-marked, 2  $\mu$ m DNA-containing vector, pYES2 [71], yielding pYES2-*PKH1*. Likewise, to express *PKH2* under *GAL1* promoter control, a 3.3 kb *BamHI-NotI* fragment containing the entire *PKH2* open reading frame was inserted into pYES2,  
10 yielding pYES2-*PKH2*.

To express mammalian PDK1 in yeast under control of the *PKH1* promoter, first, a 0.7 kb fragment corresponding to the *PKH1* promoter region was amplified by PCR from the YEplac195-*PKH1* construct using the primers 5'-GG  
15 GGT ACC GCT TGA CTC AAT TAA GGC GAC-3' (forward) and 5'-CTT CAG AGA TCA GCT TCT GCT CCA T ATT AAT GAT ATA GTA-3' (reverse), corresponding to the start of the PDK1 coding sequence (underlined). Second, a 1.4 kb fragment comprising the N-terminal sequence of PDK1 was amplified from a human PDK1 cDNA using as primers the 0.7 PCR  
20 amplification product (forward) and 5'-ACA CGA TCT CAG CCG TGT AAA A-3'(reverse), corresponding to residues 190-184 of PDK1. The 1.4 kb product was cleaved at the *KpnI* site (*italics*) and also with *HindIII*, which cleaves at an internal site in the PDK1 coding sequence. The resulting *KpnI-HindIII* fragment was used to replace a 0.5 kb segment encoding the N-terminal end of the PDK1  
25 protein either in a construct containing either the complete PDK1 coding sequence, yielding YEplac195-PDK1, or in a construct containing just the first 404 residues of the PDK1, corresponding to its catalytic domain and lacking its C-terminal PH domain, generating YEplac195-PDK1- $\Delta$ PH. To express human PDK1 under control of the *GAL1* promoter, a 2.0 kb *BglII-XbaI* fragment

containing the complete PDK1 coding sequence was inserted into pYES2, yielding pYES2-PDK1. Similarly, a 1.4 kb *Bgl*III-*Xba*I fragment containing the kinase domain of PDK1, but lacking the PH domain, was inserted into pYES2, creating pYES2- PDK1- $\Delta$ PH.

5

To express *YKR2* in yeast from a *LEU2*-marked, high-copy-number (2  $\mu$ m DNA-based) plasmid under control of the *GAL1* promoter, a 2.4 kb *Xho*I-*Hind*III fragment of genomic DNA containing the entire *YKR2* open reading frame [43] was excised from an insert in pUC18 (generously provided by Shigeo Ohno, Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) and ligated into the vector, YEp351GAL [72], that had been linearized by digestion with *Sal*I and *Hind*III, yielding pGAL-*YKR2*. An essentially identical approach was used to express *YPK1*, excised from a genomic DNA fragment (see above), yielding pGAL-*YPK1*. Alternatively, a 2.1 kb *Bam*HI-*Not*I fragment encoding an HA-tagged version of the *YPK1* coding sequence, generated by PCR, was inserted into pYES2, yielding pYES2-*YPK1*. To place the *YKR2* gene under control of its endogenous promoter on a low-copy-number (*CEN*) plasmid carrying the *URA3* gene, a 2.5 kb *Eco*RI-*Eco*RI fragment of the original *YKR2*-containing insert in pUC18 was ligated into the vector, pRS316 [64], that had been linearized with *Eco*RI, generating pYKR2(*URA3*). To generate a version of Ypk1 tagged at its C-terminal end with the c-Myc epitope recognized by the monoclonal antibody 9E10 [73], a PCR-based method for precise gene fusion [74] was performed using the *YPK1* sequence cloned in pGEM3 as one template and, as the other template, a sequence encoding the 16-residue version of the Myc epitope followed by a (His)<sub>6</sub> tag cloned in pBluescript (Stratagene), and three appropriate synthetic oligonucleotide primers: P1; T3 (Stratagene), 5'-AAT TAA CCC TCA CTA AAG GG-3', corresponding to sequences in the pBluescript vector; and, a "joiner" primer (P3), 5'-TTC AGA AAT CAA CTT TTG TTC TCT AAT GCT TCT ACC TTG C-3',

corresponding to the 3'-end of the *YPK1* coding sequence and the first several residues of the c-Myc epitope. A 2 kb *ClaI-SaII* fragment of the resulting product was used to replace the corresponding segment in the original *YPK1*-containing pGEM3 vector, yielding p*YPK1myc*. A 1.2 kb *NcoI-HindIII* fragment  
5 of p*YPK1myc* was used to replace the corresponding segment of p *GAL-YPK1*, yielding p*GAL-YPK1myc*.

To express SGK in yeast, a 1.3 kb *NcoI-EcoRI* fragment encoding a rat SGK cDNA (Webster *et al* (1993) *Mol Cell Biol* 13(4), 2031-2040) was converted to  
10 blunt ends by treatment with the Klenow fragment of *E. coli* DNA polymerase I in the presence of dNTPs and inserted behind the *ADHI* promoter in the vector pAD4M [75] that had been linearized with *SmaI*. Correct orientation of the fragment was confirmed by appropriate restriction enzyme digests. An essentially identical approach was used to express bovine  $\beta$ ARK, yielding p*ADH-*  
15  *$\beta$ ARK*, which was constructed by Henrik Dohlman (Thorner laboratory). To express PKB in yeast, a 1.5 kb *BamHI-BamHI* fragment encoding mouse c-Akt was excised from an insert in a two-hybrid bait vector, pASIIA (supplied by Zhou Songyang, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA), and ligated into YE

351GAL that had been linearized by  
20 digestion with *BamHI*, yielding p*GAL-PKB*. Alternatively, a 2.5 kb *EcoRI-XbaI* fragment encoding a human PKB cDNA [1] was inserted into pYES2, generating pYES2-PKB $\alpha$ . In addition, a 1.5 kb *EcoRI-XbaI* insert expressing a constitutively-active mutant version of PKB $\alpha$  [22], in which Thr308 and Ser473 have been replaced by Asp, was inserted into pYES2, creating pYES2-DD-  
25 PKB $\alpha$ . To express p70 S6 kinase in yeast, a 1.6 kb *XbaI-SaII* fragment encoding rat p70 S6 kinase [76] was excised from p2B4 (provided by George Thomas, Friedrich Miescher Institute, Basel, Switzerland) and inserted into YE

351GAL that had been linearized by digestion with *XbaI-SaII*, yielding p*GAL-S6K*. In addition, an N-terminal truncation, a C-terminal truncation, and a double N- and



C-terminal truncation of p70 S6 kinase (generously provided by John Blenis, Department of Cell Biology, Harvard Medical School, Boston, MA), whose constructions are described in detail elsewhere [51], were also inserted into a yeast expression vector, YEp352 [78], each under control of the methionine-repressible *MET3* promoter [78], using essentially identical methods.

#### *Site-directed mutagenesis*

To generate a catalytically-inactive ("kinase-dead") version of Pkh1 (KD-Pkh1), Asp276 (GAT) was changed to Ala (GCT). This position corresponds to a conserved residue critical for recognition of the  $Mg^{2+}$ -ATP substrate in all protein kinases [79]. Likewise, a catalytically-inactive Ypk1 derivative (KD-Ypk1) was generated by changing Asp488 (GAT) to Ala (GCT). To attempt to generate a constitutively-active *YPK1* derivative, the Pkh1-dependent phosphorylation site (Thr504) and another presumptive phosphorylation site (Thr662) that matches the consensus for phosphorylation by PDK2 protein kinase activity were both replaced by Asp codons.

#### *Expression and purification of GST-Pkh1, GST-Ypk1 and GST-SGK*

The human embryonic kidney cell line, 293, was cultured on forty 10-cm dishes, and each dish was transfected with 20  $\mu$ g of the appropriate expression construct using a modified calcium phosphate method [60]. At 24 h after transfection, each dish of cells was harvested and the cells ruptured in 1 ml of ice-cold lysis buffer, which contained 50 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium  $\beta$ -glycerol-phosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1  $\mu$ M microcystin-LR, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 10  $\mu$ g/ml leupeptin and 0.1% (by vol) 2-mercaptoethanol. The forty lysates were pooled, clarified by centrifugation at 4°C for 10 min at 13,000 x g, and the GST-fusion proteins were purified by affinity chromatography on glutathione-Sepharose [26].

Approximately 0.5 mg of each purified GST-fusion protein was obtained, snap frozen in aliquots in liquid N<sub>2</sub>, and stored at -80°C.

*Measurement of Ypk1, SGK and PKB activities*

- 5 Assay of Ypk1 was carried out in two stages. First (Stage 1), GST-Ypk1 was activated by incubation with GST-Pkh1 and Mg<sup>2+</sup>-ATP, as follows. A reaction mixture (18 µl) containing 2.5 µM PKI, 1 µM microcystin-LR, 10 mM Mg-acetate, 100 µM unlabelled ATP and 0.6 µM GST-Ypk1 was prepared in Buffer A [50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, and 0.1 % (by vol) 2-mercaptoethanol]. Reaction was initiated by addition of 2 µl of 50 nM GST-Pkh1 in Buffer A containing 1 mg/ml bovine serum albumin, and was incubated at 30°C for 30 min. Second (Stage 2), activated Ypk1 was assayed by adding 30 µl of a mixture in Buffer A containing 2.5 µM PKI, 1 µM microcystin-LR, 10 mM Mg-acetate, 100 µM [ $\gamma$ -<sup>32</sup>P]ATP (200-400 cpm/pmol) and 100 µM Crosstide (GRPRTSSFAEG) [17], a peptide phosphoacceptor substrate. After incubation for 15 min at 30°C, reaction was terminated by spotting a portion (45 µl) of each reaction mixture onto small squares of phosphocellulose paper (Whatman P81), which were washed and analysed as described [80]. Control reactions omitted either GST-Ypk1 or GST-Pkh1 and resulted in incorporation of less than 5% of the radioactivity measured in the presence of both of these proteins. One unit of GST-Ypk1 activity was defined as that amount required to catalyse phosphorylation of 1 nmole of Crosstide in 1 min. Assay of SGK and PKB activities were carried out in identical manner, except that GST-SGK and GST-PKB $\alpha$  replaced GST-Ypk1 in the first stage of the assay.

25

*Phosphorylation of GST-Ypk1, GST-PKB and GST-SGK by Pkh1*

Incubations were identical to Stage 1 of the Ypk1 assay described above, except that [ $\gamma$ -<sup>32</sup>P]ATP (500-1000 cpm/pmol) was used instead of unlabelled ATP, and reactions were terminated by adding SDS to a final concentration of 1%. The

resulting samples were resolved on 7.5% SDS-polyacrylamide gels and, after staining with Coomassie blue, analysed by autoradiography. Also, the stained band corresponding to the GST-fusion protein of interest was excised and the amount of radioactivity incorporated was quantified by liquid scintillation counting. Ability of GST-Pkh1 to phosphorylate and activate human PKB $\alpha$  was examined using methods identical to those described immediately above for the phosphorylation and activation of yeast Ypk1 by GST-Pkh1, except that reactions were performed in the presence of lipid vesicles containing various 3-phosphoinositides [26], as described in Results.

10

#### *Determination of Ypk1 substrate specificity*

GST-Ypk1 was activated with Pkh1 in vitro and incubated under standard assays conditions, as described above, except that Crosstide was replaced by 100  $\mu$ M of the peptides discussed in detail in Results. GST-PKB, derived from transfected IGF1-stimulated 293 cells [26], was assayed in parallel.

15

#### References

- 20 1. Coffey & Woodgett *Eur J Biochem.* 1991, 201:475-481.
2. Jones *et al* (1991) *Proc Natl Acad Sci. USA* 88:4171-4175.
3. Belacossa *et al* (1991) *Science* 254:244-247.
4. Franke *et al* (1995) *Cell* 81:727-736.
5. Burgering *et al* (1995) *Nature* 376:599-602.
- 25 6. Kohn *et al* (1995) *EMBO J* 14:4288-4295.
7. Alessi & Cohen (1998) *Curr Opin Genet Dev* 8:55-62.
8. Downward (1998) *Curr Opin Cell Biol* 10:262-267.
9. Barthel *et al* (1997) *Endocrinol* 138:3559-3562.
10. Cichy *et al* (1998) *J Biol Chem* 273:6482-6487.

11. Cheng *et al* (1992) *Proc Natl Acad Sci USA* **89** : 9267-9271.
12. Cheng *et al* (1996) *Proc Natl Acad Sci USA* **93**: 3636-3641.
13. Bellacosa *et al* (1995) *Intl. J. Cancer* 1995, **64**: 280-285.
14. del Peso *et al* (1997) *Science* **278**:687-689.
- 5 15. Dudek *et al* (1997) *Science* **275**: 661-665.
16. Alessi *et al* (1996) *FEBS Lett* **399**: 333-338.
17. Cross *et al* (1995) *Nature* **378**:785-789.
18. van Weeren *et al* (1998) *J Biol Chem* **273** :13150-13156.
19. Deprez *et al* (1997) *J Biol Chem* **272**:17269-17275.
- 10 20. Welsh *et al* (1998) *FEBS Lett.* **421**:125-130.
21. Datta *et al* (1997) *Cell* **91**:231-241.
22. Alessi *et al* (1996) *EMBO J* **15**:6541-6551.
23. Kohn *et al* (1996) *J Biol Chem* **271**: 21920-21926.
24. Pearson *et al* (1995) *EMBO J* **14**: 5278-5287.
- 15 25. Alessi *et al* (1998) *Curr Biol* **8**:69-81.
26. Alessi *et al* (1997) *Curr Biol* **7**: 261-269.
27. Alessi *et al* (1997) *Cur Biol* **7**:776-789.
28. Kavanaugh *et al* (1996) *Curr Biol* **6**:438-445.
29. LeGood *et al* (1998) *Science* **281**:2042-2045.
- 20 30. James *et al* (1996) *Biochem J* **315**:709-713.
31. Frech *et al* (1996) *J Biol Chem* **272**:8474-8478.
32. Stephens *et al* (1988) *Science* **279**:710-714.
33. Currie *et al* (1999) *Biochem J* **337**( Pt 3), 575-83
34. Pullen *et al* (1998) *Science* **279**:707-710.
- 25 35. Le Good *et al* (1998) *Science* **281**(5385), 2042-5
36. Kobayashi & Cohen P (1999) *Biochem J* **339** ( Pt 2), 319-28
37. Jacq *et al* (1997) *Nature* **387**SS:75-78.
38. Dujon *et al* (1997) *Nature* **387**SS: 98-102.

39. Mumberg *et al* (1994) *Nucleic Acid Res* 22:5767-5768.
40. Boeke *et al* (1986) *Mol Gen Genet* 197:345-346
41. Maurer *et al* (1988) *DNA* 7:469-474.
42. Chen *et al* (1993) *Mol Gen Genet* 236:443-447.
- 5 43. Kubo *et al* (1989) *Gene* 76:177-180.
44. Toda *et al* (1988) *Genes Dev* 2:517-527.
45. Levin *et al* (1990) *Cell* 62:213-224.
46. Webster *et al* (1993) *Mol Cell Biol* 13:2031-2040.
47. Benovic *et al* (1991) *J Biol Chem* 266:14939-14946.
- 10 48. Bennetzen *et al* (1982) *J Biol Chem* 257:3018-3025.
49. Rose *et al* (1990) *Methods Enzymol* 185:234-279.
50. Proud *et al* (1996) *Trends Biochem Sci* 21:181-185.
51. Cheatham *et al* (1995) *Proc. Natl. Acad. Sci. USA*, 92:1696-1700.
52. Hawkins *et al* (1993) *J Biol Chem* 268:3374-3383.
- 15 53. Dove *et al* (1997) *Nature* 390:187-192.
54. DeWald *et al* (1997) *Biochem Soc Trans* 25:1141-1146.
55. Hunter & Plowman (1997) *Trends Biochem Sci* 22:18-22.
56. Mellor & Parker (1998) *Biochem J* 332:281-292.
57. Xue *et al* (1998) *EMBO J* 17:1996-2007.
- 20 58. Sherman F, Fink GR, Hicks JB: **Methods in Yeast Genetics**. Cold Spring Harbor NY: Cold Spring Harbor Laboratory Press; 1986
59. Guthrie C, Fink GR (Editors): **Guide to Yeast Genetics and Molecular Biology**, New York NY: Academic Press, Inc.; 1991
60. Sambrook J, Fritsch EF, Maniatis T: **Molecular cloning: a laboratory**
- 25 **manual**, Cold Spring Harbor NY: Cold Spring Harbor Laboratory Press; 1989
61. Schiestl & Gietz (1989) *Curr. Genet.* 16:339-346.
62. Gietz *et al* (1992) *Nucleic Acid Res* 20:1425.
63. Lorenz *et al* (1995) *Gene* 158:113-117.

64. Sikorski & Hieter (1989) *Genetics* 122:19-27.
65. Schnieders, EA: *Ypk1 and Ykr2*. Appendix, Doctoral Dissertation, University of California, Berkeley, CA, 1996, pp.158-185.
66. Muhlrads *et al* (1992) *Yeast* 8:79-82.
- 5 67. Staples & Dieckmann (1993) *Genetics* 135:981-991.
68. Hoffman & Winston (1987) *Gene* 57:267-272.
69. Sanchez *et al* (1994) *Nature* 372:794-798.
70. Gietz & Sugino (1988) *Gene* 74:527-534.
71. Ramer *et al* (1992) *Proc Natl Acad Sci USA* 89:11589-11593.
- 10 72. Benton *et al* (1994) *J Cell Biol* 127:623-639.
73. Evan *et al* (1985) *Mol Cell Biol* 5:3610-3616.
74. Yon & Fried (1989) *Nucleic Acids Res* 17: 4895.
75. Martin *et al* (1990) *Cell* 63:843-849.
76. Kozma *et al* (1990) *Proc Natl Acad Sci USA* 87:7365-7369.
- 15 77. Hill *et al* (1986) *Yeast* 2:163-167.
78. Cherest *et al* (1987) *Mol. Gen. Genetics* 210:307-313.
79. Taylor SS & Radzio-Andzelm (1994) *Structure* 2:345-355.
80. Alessi *et al* (1995) *Methods Enzymol* 255:279-290.
81. Julius *et al* (1984) *Cell* 36:309-318.
- 20 82. Ma *et al* (1995) *Mol Biol Cell* 6:889-909.
83. Currie *et al* (1999) *Biochem J* 337( Pt 3), 575-83

**Table 1.** *Saccharomyces cerevisiae* strains used.

	Strain	Genotype	Source
25	AYS927	<i>MATa/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100 ssd1-d2/ssd1-d2</i>	M.J.R. Stark
	AC200	<i>AYS927 PKH2/pkh2Δ::HIS3</i>	This study

	AC201	AYS927 <i>PKH1/pkh1Δ::TRP1</i>	This study
	AC303	<i>MATa pkh2Δ::HIS3</i> (derived from AC200)	This study
	AC301	<i>MAT pkh1Δ::TRP1</i> (derived from AC201)	This study
	AC306	<i>MATa/MATα PKH1/pkh1Δ::TRP1 PKH2/pkh2Δ::HIS3</i>	This study
5		(AC303 X AC301)	
	YPH499	<i>MATa ade2-101<sup>oc</sup> his3-Δ200 leu2-Δ1 lys2-801<sup>am</sup></i>	[64]
		<i>trp1-Δ1 ura3-52</i>	
	YPH500	<i>MATα</i> otherwise isogenic to YPH499	[64]
	YES1	YPH499 <i>ykr2-Δ1::TRP1</i>	This study
			This study
10	YES5	YPH500 <i>ypk1-Δ1::HIS3</i>	
	YES7	<i>MATa/MATα YPK1/ypk1Δ::HIS3 YKR2/ykr2Δ::TRP1</i>	This study
		(YES1 X YES5)	
	YPT28	<i>MATa ypk1Δ::HIS3 ykr2Δ::TRP1 [pYKR2(URA3)]</i>	This study
	YPT40	<i>MATa ypk1-1<sup>ts</sup> ykr2Δ</i>	This study

**Table 2. Substrate selectivity of yeast Ypk1 and mammalian PKB $\alpha$ .**

Peptide	<u>Relative Rate of Phosphorylation</u> (at 0 .1 mM)	
	GST-Ypk1	GST-PKB $\alpha$
5		
1	GRPRTSSFAEG	[100]
2	RPRTSSF	139
3	KRPRTSSF	27
4	RPKTSSF	30
10	5 RPRTSAF	0*
6	PRTSSF	0*
7	RPRTSS	2*
8	KKRNRTL SVA	157
15	9 KKKNRTL SVA	13
10	KKRNKTL SVA	25
11	RPRTSSF	139
12	RPRTSSV	108
13	RPRTSSL	130
20	14 RPRTSSA	70
15	RPRTSSK	67*
16	RPRTSSE	63

25 \*Data taken from Ref. 16.

### Example 2: screening assay for inhibitors of human PDK1

The effect of compounds on parental (wild-type with respect to Pkh1 and/or Pkh2) *S. cerevisiae* cells and *S. cerevisiae* cells in which the endogenous Pkh1 and Pkh2 genes have been inactivated and in which human PDK1 is expressed

30



under control of the Pkh1 promoter, are compared. Compounds that affect the growth of the PDK1-expressing cells but not the Pkh1/Pkh2 expressing (wild-type) cells are selected and may be used to design further compounds for manufacture and test, in order to develop a structure-activity relationship (SAR).

5

The compounds for test may be selected on the basis of known kinase inhibitory activity or other known property, or may be part of a library of synthetic or natural molecules that may be screened in a "lead generation" screening project.

10 **Example 3: screening assay for inhibitors of PDK1 from a pathogenic source**

The effect of compounds on parental (wild-type with respect to Pkh1 and/or Pkh2) *S. cerevisiae* cells and *S. cerevisiae* cells in which the endogenous Pkh1 and Pkh2 genes have been inactivated and in which *Candida* Pkh1 or Pkh2 is expressed under control of the Pkh1 promoter, are compared. Compounds that affect the growth of the *Candida* Pkh1 or Pkh2-expressing cells but not the wild-type Pkh1/Pkh2 expressing cells are selected and may be used to design further compounds for manufacture and test, in order to develop a structure-activity relationship (SAR).

20

The compounds for test may be selected on the basis of known kinase inhibitory activity or other known property, or may be part of a library of synthetic or natural molecules that may be screened in a "lead generation" screening project.

25 **Example 4: Identification of *Candida* genes related to *S. cerevisiae* Pkh1, Pkh2, Ypk1 and Ykr2 genes.**

The *Candida* sequence database only allows DNA searches of the *Candida* data. Therefore, for each of the 4 *S. cerevisiae* sequences identified and characterised,

as described in example 1 (Pkh1, Pkh2, Ypk1, Ykr2), the coding sequence for each *S. cerevisiae* gene was used to search the Candida database.

5 The best matches in each case were selected and compared with proteins in the Genbank database. With one exception, this comparison identified the two related *S. cerevisiae* genes (ie Pkh1/Pkh2 or Ypk1/Tkr2) as the most closely related proteins.

10 The *Candida albicans* database entries selected for further study on the basis of the four coding sequence searches were:

384230A10.s4.seq (search 1)  
384362E11.s1.seq (search 1 and search 2)  
384286E10.s1.seq (search 1 and search 2)  
396076E03.s2.seq (search 3 and search 4)  
15 384194F08.s1.seq (search 3 and search 4)

There appear to be fragments of three Candida genes in the database related very closely to *S. cerevisiae* Pkh1, Pkh2, Ypk1 and Ykr2.

20 384194F08.s1.seq and 396076E03.s2.seq appear to be closely related to Pkh1 and Pkh2.

384362E11.s1.seq and 384286E10.s1.seq appear to be closely related to Ypk1 and Ykr2/Ypk2.

25

Full-length coding sequences may be determined for the genes from which the above nucleotide sequences are derived by well known techniques, which may include further database interrogation and/or techniques of molecular biology,  
30 which may include PCR or library-based cloning techniques.

09/868118  
PCT/GB99/04228

WO 00/36135

115

## Search 1

## Blast Results

5

## Smallest

## Sum

10

## High

## Probability

Sequences producing High-scoring Segment Pairs:

Score P(N) N

15

384230A10.s4.seq 635 3.2e-43 1

384362E11.s1.seq 595 3.8e-40 1

CAPKC1 C.albicans gene for protein kinase C. 490 5.2e-37 2

384286E10.s1.seq 411 1.5e-24 1

384201A03.s2.seq 363 1.4e-20 1

385002F05.x1.seq 350 1.9e-19 1

20

396143F08.s3.seq 307 7.1e-16 1

CAU73457 Candida albicans Cst20p (CST20) gene, complete ... 240 4.4e-10 1

YSASTPK Candida albicans serine/threonine protein kinase... 240 4.4e-10 1

265251G07.x1.seq 233 1.4e-09 1

265251H07.x1.seq 233 1.4e-09 1

25

385040C04.x1.seq 229 2.9e-09 1

386003B12.x1.seq 218 2.5e-08 1

396076E03.s2.seq 189 7.1e-06 1

384281A03.s1.seq 174 0.00012 1

384194F08.s1.seq 172 0.00017 1

30

384253B09.s1.seq 165 0.00069 1

384329A05.s1.seq 164 0.00084 1

265251H06.x1.seq 162 0.0012 1

265273H11.y1.seq 157 0.0032 1

265266H04.x1.seq 155 0.0049 1

35

384068G08.s1.seq 152 0.0069 1

396170G09.s1.seq 151 0.0096 1

## Search 2

## Blast Results

40

## Smallest

45

## Sum

## High

## Probability

Sequences producing High-scoring Segment Pairs:

Score P(N) N

50

384362E11.s1.seq 586 2.2e-49 2

384286E10.s1.seq 497 8.7e-32 1

CAU73457 Candida albicans Cst20p (CST20) gene, complete ... 287 5.3e-14 1

YSASTPK Candida albicans serine/threonine protein kinase... 287 5.3e-14 1

265251G07.x1.seq 264 3.6e-12 1

55

265251H07.x1.seq 262 5.3e-12 1

265251H06.x1.seq 242 2.4e-10 1

265266H04.x1.seq 226 5.6e-09 1

385040C04.x1.seq 219 2.0e-08 1

YSASTKIN Candida albicans (clone pKB66) serine/threonine... 185 1.6e-05 1

	CAU72980 Candida albicans Map kinase kinase (STE7) gene,...	185	1.7e-05	1
	386003B12.x1.seq	181	3.2e-05	1
	385081C04.x1.seq	164	0.00079	1
5	396039B07.s1.seq	159	0.0021	1
	384281A03.s1.seq	156	0.0039	1

## Search 3

## Blast Results

10

## Smallest

## Sum

15

## Probability

## High

Sequences producing High-scoring Segment Pairs:

Score P(N) N

20	396076E03.s2.seq	636	6.9e-85	2
	384194F08.s1.seq	674	1.5e-46	1
	384254G10.s1.seq	266	3.8e-12	1
	385051D09.x1.seq	244	2.1e-10	1
	384022B04.s1.seq BCK1	244	2.6e-10	1
25	CAU73457 Candida albicans Cst20p (CST20) gene, complete ...	229	5.9e-09	1
	YSASTPK Candida albicans serine/threonine protein kinase...	229	5.9e-09	1
	384201A03.s2.seq	220	2.5e-08	1
	384168E05.s2.seq	216	3.1e-08	1
	CAU87996 Candida albicans CLA4 protein kinase homolog ge...	218	4.8e-08	1
30	396039B07.s1.seq	195	3.3e-06	1
	384282H11.s1.seq	187	1.5e-05	1
	385066D07.x1.seq	171	0.00033	1
	265235H07.y1.seq	169	0.00047	1
	396039H02.s1.seq	162	0.0012	1
35	396256F10.s1.seq	164	0.0013	1
	384102B12.s1.seq	163	0.0016	1
	265091D04.x1.seq PAK1	159	0.0035	1
	Unp-F1-F17E2-Reverse (-) Assignment:KIN3	156	0.0061	1
	385009G05.x1.seq	154	0.0091	1
40	384179F07.s1.seq	153	0.011	1
	265001E11.r2.seq STE11	150	0.020	1

## Search 4

## Blast Results

45

## Smallest

## Sum

50

## Probability

## High

Sequences producing High-scoring Segment Pairs:

Score P(N) N

55	396076E03.s2.seq	636	6.9e-85	2
	384194F08.s1.seq	674	1.5e-46	1
	384254G10.s1.seq	266	3.8e-12	1
	385051D09.x1.seq	244	2.1e-10	1
	384022B04.s1.seq BCK1	244	2.6e-10	1
	CAU73457 Candida albicans Cst20p (CST20) gene, complete ...	229	5.9e-09	1

	YASTPK Candida albicans serine/threonine protein kinase...	229	5.9e-09	1
	384201A03.s2.seq	220	2.5e-08	1
	384168E05.s2.seq	216	3.1e-08	1
5	CAU87996 Candida albicans CLA4 protein kinase homolog ge...	218	4.8e-08	1
	396039B07.s1.seq	195	3.3e-06	1
	384282H11.s1.seq	187	1.5e-05	1
	385066D07.x1.seq	171	0.00033	1
	265235H07.y1.seq	169	0.00047	1
10	396039H02.s1.seq	162	0.0012	1
	396256F10.s1.seq	164	0.0013	1
	384102B12.s1.seq	163	0.0016	1
	265091D04.x1.seq PAK1	159	0.0035	1
	Unp-F1-F17E2-Reverse (-) Assignment:KIN3	156	0.0061	1
15	385009G05.x1.seq	154	0.0091	1
	384179F07.s1.seq	153	0.011	1
	265001E11.r2.seq STE11	150	0.020	1

### Genbank search results using 384194F08.s1.seq (search 3 and search 4)

#### 20 Blast Results

				Smallest Sum	
		Reading	High	Probability	
	Sequences producing High-scoring Segment Pairs:	Frame	Score	P(N)	N
	gi 663254 (Z48149) probable protein kinase [Saccharom...	-3	452	4.0e-55	1
	gi 1419952 (Z74842) ORF YOL100w [Saccharomyces cerevis...	-3	452	4.0e-55	1
30	gi 1431588 (X99280) protein kinase [Schizosaccharomyce...	-3	441	1.7e-54	1
	gi 927745 (U33050) Ydr490cp; CAI: 0.11 [Saccharomyces...	-3	446	2.1e-54	1
	gi 2407613 (AF017995) 3-phosphoinositide dependent pro...	-3	232	4.0e-48	2
	gi 2505936 (Y15056) PkB kinase [Homo sapiens]	-3	232	4.0e-48	2
	gi 2665356 (Y15748) PkB kinase [Rattus norvegicus]	-3	232	4.1e-48	2
35	gi 2832892 (AL021730) hypothetical protein kinase phos...	-3	393	1.8e-47	1
	gi 927730 (U33050) Ydr466wp; CAI: 0.11 [Saccharomyces...	-3	250	3.5e-47	3
	gi 2980851 (Y07908) serine/threonine protein kinase [D...	-3	351	3.3e-41	1
	gi 1000069 (U15210) rac-alpha serine/threonine kinase ...	-3	184	2.0e-31	2
40	gi 1673493 (Z81140) W10G6.2 [Caenorhabditis elegans]	-3	277	4.0e-31	1
	gi 294637 (L01624) serine/threonine protein kinase [R...	-3	277	4.1e-31	1
	gi 1834511 (Y10032) serine/threonine protein kinase [H...	-3	276	5.6e-31	1
	gi 2463201 (AJ000512) serine/threonine protein kinase ...	-3	276	5.6e-31	1
	gi 189967 (M80335) protein kinase A-alpha [Homo sapiens]	-3	156	5.9e-29	2
45	gi 2632252 (Y12464) serine/threonine kinase [Sorghum b...	-3	161	2.3e-28	2
	gi 1401040 (D49836) RAC-PK gamma [Rattus norvegicus]	-3	167	2.5e-28	2
	gi 1890142 (D83380) catalytic subunit of cAMP-dependen...	-3	167	4.1e-28	2
	gi 773642 (U23730) vinclozolin resistance protein [Us...	-3	172	6.5e-28	2
	gi 2213667 (U83459) Rim15p [Saccharomyces cerevisiae]	-3	255	8.5e-28	1
50	gi 836721 (D50617) YFL033C [Saccharomyces cerevisiae]	-3	255	8.5e-28	1
	gi 465398 (D28577) Protein kinase C [Mus musculus]	-3	253	1.2e-27	1
	gi 432274 (L18964) protein kinase C iota [Homo sapiens]	-3	253	1.2e-27	1
	gi 598225 (L33881) protein kinase C iota [Homo sapiens]	-3	253	1.2e-27	1
	gi 507141 (M81709) cAMP-dependent protein kinase cata...	-3	166	1.2e-27	2
55	gi 2253148 (Z82096) ZK909.2a [Caenorhabditis elegans]	-3	162	1.2e-27	2
	gi 304273 (L17008) cAMP-dependent protein kinase [Bla...	-3	166	1.4e-27	2
	gi 2253149 (Z82096) ZK909.2b [Caenorhabditis elegans]	-3	162	1.5e-27	2
	gi 853791 (Z34989) Ndr protein kinase [Caenorhabditis...	-3	178	1.7e-27	2
	gi 2113796 (X94399) cAMP-dependent protein kinase cata...	-3	159	1.9e-27	2
60	gi 56916 (X68400) protein kinase [Rattus norvegicus]	-3	251	2.4e-27	1
	gi 220527 (D90242) nPKC-eta [Mus musculus]	-3	251	2.4e-27	1

	gi 665540	(U15983)	cAMP-dependent protein kinase cata...	-3	160	3.1e-27	2
	gi 189989	(M55284)	protein kinase C-L [Homo sapiens]	-3	250	3.3e-27	1
	gi 806542	(Z49233)	calcium-stimulated protein kinase ...	-3	249	4.2e-27	1
5	gi 473894	(U08622)	cAMP-dependent protein kinase [Sch...	-3	169	4.3e-27	2
	gi 484305	(D23667)	catalytic subunit of the cAMP-depe...	-3	169	4.3e-27	2
	gi 853820	(Z35103)	Ndr protein kinase [Drosophila mel...	-3	164	4.5e-27	2
	gi 2632254	(Y12465)	serine/threonine kinase [Sorghum b...	-3	163	5.6e-27	2
	gi 1911	(X05998)	C-beta subunit (338 AA) [Sus scrofa]	-3	155	6.5e-27	2
10	gi 516040	(U12335)	cAMP-dependent protein kinase cata...	-3	159	6.6e-27	2
	gi 35479	(X07767)	protein kinase catalytic subunit t...	-3	156	7.5e-27	2
	gi 1487920	(Z75953)	F57F5.5 [Caenorhabditis elegans]	-3	247	7.6e-27	1
	gi 3005054	(AF041843)	protein kinase Ukclp [Ustilago m...	-3	177	8.4e-27	2
	gi 392435	(U00181)	protein kinase C [Caenorhabditis e...	-3	247	8.7e-27	1
	gi 2304746	(A52140)	HUMAN NDR [unidentified]	-3	172	8.9e-27	2
15	gi 854170	(Z35102)	Ndr protein kinase [Homo sapiens]	-3	172	8.9e-27	2
	gi 173011	(M17073)	cAMP-dependent protein kinase subu...	-3	160	9.7e-27	2
	gi 4625	(Y00694)	put. kinase (AA 1-380) [Saccharomy...	-3	160	9.7e-27	2
	gi 1370422	(Z73559)	ORF YPL203w [Saccharomyces cerevis...	-3	160	9.7e-27	2
20	gi 191175	(M63311)	cAMP-dependent protein kinase alph...	-3	156	1.0e-26	2
	gi 156233	(M37120)	cAMP-dependent protein kinase cata...	-3	156	1.3e-26	2
	gi 1086654	(U41016)	coded for by C. elegans cDNA CEESC...	-3	178	1.6e-26	2
	gi 288120	(X53261)	cAMP-dependent protein kinase cata...	-3	153	1.7e-26	2
	gi 2760821	(L06196)	serine/threonine protein kinase [E...	-3	174	1.7e-26	2
25	gi 1050791	(X92517)	N1727 gene product [Saccharomyces ...]	-3	175	1.7e-26	2
	gi 1302128	(Z71437)	ORF YNL161w [Saccharomyces cerevis...	-3	175	1.7e-26	2
	gi 3043598	(AB011109)	KIAA0537 protein [Homo sapiens]	-3	196	1.9e-26	2
	gi 1853976	(D31773)	protein kinase [Schizosaccharomyce...	-3	245	2.0e-26	1
	gi 200367	(M12303)	cAMP-dependent protein kinase cata...	-3	158	2.0e-26	2
30	gi 387513	(M19960)	cAMP-dependent protein kinase alph...	-3	158	2.0e-26	2
	gi 155792	(M94884)	protein kinase C [Aplysia californ...	-3	244	2.3e-26	1
	gi 178326	(M95936)	protein serine/threonine kinase [H...	-3	160	2.5e-26	2
	gi 191177	(M63312)	cAMP-dependent protein kinase beta...	-3	152	2.7e-26	2
	gi 200387	(J02626)	cAMP-dependent protein kinase beta...	-3	152	2.7e-26	2
35	gi 220704	(D10770)	cAMP-dependent protein kinase cata...	-3	152	2.7e-26	2
	gi 163532	(J02647)	protein kinase beta-catalytic subu...	-3	151	2.7e-26	2
	gi 189983	(M34181)	cAMP-dependent protein kinase cata...	-3	151	2.7e-26	2
	gi 337491	(M77198)	rac protein kinase-beta [Homo sapi...	-3	160	3.0e-26	2
	gi 2911458	(AF046921)	cAMP-dependent protein kinase ca...	-3	164	3.1e-26	2
40	gi 2244814	(Z97336)	protein kinase [Arabidopsis thaliana]	-3	242	3.1e-26	1
	gi 56912	(X57986)	cAMP-dependent protein kinase subu...	-3	156	3.8e-26	2
	gi 162787	(M60482)	cAMP-dependent protein kinase II-b...	-3	151	4.0e-26	2
	gi 165563	(M20014)	protein kinase C [Oryctolagus cuni...	-3	242	4.4e-26	1
	gi 35495	(X65293)	protein kinase C epsilon [Homo sap...	-3	242	4.4e-26	1
45	gi 2605804	(AF028009)	protein kinase C epsilon [Mus mu...	-3	242	4.4e-26	1
	gi 633	(X67154)	protein kinase [Bos taurus]	-3	156	5.2e-26	2
	gi 942578	(U22445)	serine/threonine kinase [Mus muscu...	-3	157	6.5e-26	2
	gi 157052	(M18655)	cAMP-dependent protein kinase cata...	-3	155	7.3e-26	2
	gi 7807	(X16969)	catalytic subunit [Drosophila mela...	-3	155	7.3e-26	2
50	..						
	..						

Genbank results using 384230A10.s4.seq (search 1)

## 55 Blast Results

			Smallest
			Sum
60	Sequences producing High-scoring Segment Pairs:	Reading Frame	High Probability P(N)
		Score	N

	gi 1050791	(X92517)	N1727 gene product [Saccharomyces ...	+3	921	8.3e-137	3
	gi 1302128	(Z71437)	ORF YNL161w [Saccharomyces cerevis...	+3	921	8.3e-137	3
5	gi 3005054	(AF041843)	protein kinase Ukclp [Ustilago m...	+3	817	3.0e-117	2
	gi 2443511	(AF009512)	protein kinase Orb6p [Schizosacc...	+3	769	8.7e-115	2
	gi 1870020	(X97657)	serine/threonine kinase [Neurospor...	+3	725	1.2e-102	2
	gi 641979	(U14989)	kinase [Colletotrichum trifolii]	+3	733	4.5e-100	2
	gi 1870019	(X97657)	serine/threonine kinase [Neurospor...	+3	725	1.3e-99	2
10	gi 2304746	(A52140)	HUMAN NDR [unidentified]	+3	647	1.5e-93	2
	gi 854170	(Z35102)	Ndr protein kinase [Homo sapiens]	+3	647	1.5e-93	2
	gi 853820	(Z35103)	Ndr protein kinase [Drosophila mel...	+3	629	2.7e-92	2
	gi 2304742	(A52135)	D. MELANOGASTER NDR [unidentified]	+3	372	1.8e-89	3
	gi 1086654	(U41016)	coded for by C. elegans cDNA CEESC...	+3	628	2.0e-85	2
15	gi 853791	(Z34989)	Ndr protein kinase [Caenorhabditis...	+3	618	1.1e-80	1
	gi 506534	(X71057)	protein kinase [Nicotiana tabacum]	+3	435	1.1e-74	3
	gi 3135270	(AC003058)	putative protein kinase [Arabido...	+3	533	5.8e-74	2
	gi 457709	(Z30330)	protein kinase [Spinacia oleracea]	+3	533	6.3e-74	2
	gi 1914507	(Z81594)	T20F10.1 [Caenorhabditis elegans]	+3	575	1.9e-72	1
	gi 903942	(U29608)	LATS [Drosophila melanogaster]	+3	466	2.0e-71	3
20	gi 755008	(L39837)	tumor suppressor [Drosophila melan...	+3	466	2.0e-71	3
	gi 457689	(Z30329)	protein kinase [Mesembryanthemum c...	+3	548	1.4e-69	1
	gi 1200533	(M28500)	protein kinase [Euplotes crassus]	+3	469	5.0e-60	1
	gi 1200509	(U47679)	protein kinase [Euplotes crassus]	+3	469	3.5e-58	1
25	gi 1276901	(U43195)	Rho-associated, coiled-coil contai...	+3	300	3.7e-54	2
	gi 1514696	(U58512)	Rho-associated, coiled-coil contai...	+3	300	3.7e-54	2
	gi 1438567	(U61266)	Rho-associated kinase beta [Rattus...	+3	300	3.8e-54	2
	gi 2736153	(AF021936)	myotonic dystrophy kinase-relate...	+3	280	1.6e-51	2
	gi 181605	(L08835)	myotonic dystrophy kinase [Homo sa...	+3	271	2.2e-51	2
30	gi 181606	(L08835)	myotonic dystrophy kinase [Homo sa...	+3	271	2.2e-51	2
	gi 976145	(L00727)	myotonin-protein kinase, Form VIII...	+3	271	2.4e-51	2
	gi 1061299	(Z67757)	unknown [Schizosaccharomyces pombe]	+3	422	2.6e-51	1
	gi 186756	(M94203)	protein kinase [Homo sapiens]	+3	271	3.0e-51	2
	gi 976143	(L00727)	myotonin-protein kinase, Form V [H...	+3	271	3.2e-51	2
35	gi 976146	(L00727)	myotonin-protein kinase, Form VII ...	+3	271	3.5e-51	2
	gi 307177	(L19268)	protein kinase [Homo sapiens]	+3	271	3.8e-51	2
	gi 181603	(L08835)	myotonic dystrophy kinase [Homo sa...	+3	271	3.8e-51	2
	gi 633865	(S72883)	myotonin protein kinase, MtPK=thym...	+3	271	3.8e-51	2
	gi 181604	(L08835)	myotonic dystrophy kinase [Homo sa...	+3	271	3.9e-51	2
40	gi 976147	(L00727)	myotonin-protein kinase, Form VI [...	+3	271	4.0e-51	2
	gi 976144	(L00727)	myotonin-protein kinase, Form I [H...	+3	271	4.1e-51	2
	gi 1384133	(U38481)	ROK-alpha [Rattus norvegicus]	+3	285	5.7e-51	2
	gi 1326078	(U36909)	Rho-associated kinase [Bos taurus]	+3	285	5.7e-51	2
	gi 1514698	(U58513)	Rho-associated, coiled-coil contai...	+3	285	5.7e-51	2
45	gi 556903	(Z21503)	DM protein kinase [Mus musculus]	+3	268	6.8e-51	2
	gi 1695873	(U59305)	ser-thr protein kinase PK428 [Homo...	+3	266	8.2e-51	2
	gi 563526	(Z38015)	myotonic dystrophy protein kinase ...	+3	268	1.0e-50	2
	gi 171380	(M62506)	putative [Saccharomyces cerevisiae]	+3	402	1.4e-50	2
	gi 1938422	(U97001)	Similar to serine/threonine-protei...	+3	253	2.6e-50	3
50	gi 2982220	(AF037073)	Rho-associated kinase alpha [Xen...	+3	283	3.8e-50	2
	gi 1323137	(Z72877)	ORF YGR092w [Saccharomyces cerevis...	+3	413	4.4e-50	1
	..						
	..						

Genbank search results using 384286E10.s1.seq (search 1 and search 2)

55

#### Blast Results

60

WARNING: -hspmax 100 was exceeded with 1 of the database sequences, with as many as 139 HSPs being found at one time.

5	Sequences producing High-scoring Segment Pairs:	Reading Frame	High Score	Smallest Sum Probability	
				P(N)	N
10	gi 295681 (M24929) protein kinase [Saccharomyces cere...	+3	284	5.0e-32	1
	gi 817862 (Z49702) Ypk2p [Saccharomyces cerevisiae]	+3	284	5.0e-32	1
15	gi 172181 (M21307) protein kinase [Saccharomyces cere...	+3	280	1.8e-31	1
	gi 486213 (Z28126) ORF YKL126w [Saccharomyces cerevis...	+3	280	1.8e-31	1
20	gi 2911462 (AF046923) serine/threonine protein kinase ...	+3	194	1.7e-20	2
	gi 458284 (U05811) serine/threonine protein kinase [T...	+3	193	5.0e-20	2
25	gi 206181 (M18330) protein kinase C delta subspecies ...	+3	175	2.6e-15	1
	gi 189510 (M60725) p70 ribosomal S6 kinase alpha-II [...	+3	174	3.3e-15	1
30	gi 206840 (M57428) S6 kinase [Rattus norvegicus]	+3	174	3.3e-15	1
	gi 1562 (X54415) G3 serine/threonine kinase [Orycto...	+3	174	3.4e-15	1
35	gi 189508 (M60724) p70 ribosomal S6 kinase alpha-I [H...	+3	174	3.4e-15	1
	gi 2222775 (Y12002) protein kinase C homologue [Neuros...	+3	173	5.7e-15	1
40	gi 200381 (M69042) [Mouse protein kinase C delta mRNA...	+3	172	7.2e-15	1
	gi 53437 (X60304) protein kinase [Mus musculus]	+3	172	7.2e-15	1
45	gi 501075 (U10016) protein kinase C [Trichoderma reesei]	+3	172	8.0e-15	1
	gi 2687849 (Y15839) protein kinase C [Cochliobolus het...	+3	169	2.2e-14	1
50	gi 507900 (U10549) protein kinase C [Aspergillus niger]	+3	168	3.1e-14	1
	gi 206842 (M58340) S6 protein kinase [Rattus norvegicus]	+3	165	7.3e-14	1
55	gi 1136302 (D38108) protein kinase [Schizosaccharomyce...	+3	162	1.1e-13	2
	gi 165563 (M20014) protein kinase C [Oryctolagus cuni...	+3	161	3.1e-13	1
60	gi 35495 (X65293) protein kinase C epsilon [Homo sap...	+3	161	3.1e-13	1
	gi 206183 (M18331) protein kinase C epsilon subspecie...	+3	160	4.3e-13	1
65	gi 2605804 (AF028009) protein kinase C epsilon [Mus mu...	+3	159	6.0e-13	1
	gi 832908 (X81142) protein kinase C [Candida albicans]	+3	159	6.5e-13	1
70	gi 558100 (L07032) protein kinase C-theta [Homo sapiens]	+3	156	1.6e-12	1
	gi 558099 (L01087) protein kinase C-theta [Homo sapiens]	+3	156	1.6e-12	1
75	gi 458923 (U00029) Sch9p: cAMP-dependent protein kina...	+3	156	1.7e-12	1
	gi 4426 (X12560) SCH9 protein (AA 1-824) [Saccharom...	+3	156	1.7e-12	1
80	gi 5279 (X57629) Sch9 [Saccharomyces cerevisiae]	+3	156	1.7e-12	1
	gi 1778590 (U82935) protein kinase C2 A isoform [Caeno...	+3	150	2.5e-12	2
85	gi 1000069 (U15210) rac-alpha serine/threonine kinase ...	+3	151	2.6e-12	2
	gi 214789 (M20188) S6 kinase II beta [Xenopus laevis]	+3	154	3.1e-12	1
90	gi 3114960 (Y13104) Protein kinase C-related kinase (P...	+3	149	3.4e-12	2
	gi 173427 (L07637) protein kinase C [Schizosaccharomy...	+3	154	3.4e-12	1
95	gi 303941 (D14338) protein kinase [Schizosaccharomyce...	+3	154	3.4e-12	1
	gi 401772 (L07598) ribosomal protein S6 kinase 2 [Hom...	+3	152	5.9e-12	1
100	gi 220527 (D90242) nPKC-eta [Mus musculus]	+3	152	6.3e-12	1
	gi 1033033 (X85106) ribosomal S6 kinase [Homo sapiens]	+3	152	6.3e-12	1
105	gi 551556 (M28488) ribosomal protein S6 kinase [Gallu...	+3	152	6.4e-12	1
	gi 56916 (X68400) protein kinase [Rattus norvegicus]	+3	151	8.7e-12	1
110	..				
115	..				

50 Genbank search results using 384362E11.s1.seq (search 1 and search 2)

# Blast Results

55	Sequences producing High-scoring Segment Pairs:	Reading Frame	High Score	Smallest Sum Probability	
				P(N)	N
60	gi 486213 (Z28126) ORF YKL126w [Saccharomyces cerevis...	+2	391	6.6e-65	3
	gi 172181 (M21307) protein kinase [Saccharomyces cere...	+2	387	2.4e-64	3
	gi 295681 (M24929) protein kinase [Saccharomyces cere...	+2	380	5.8e-63	3



	gi 817862	(Z49702)	Ypk2p [Saccharomyces cerevisiae]	+2	380	5.8e-63	3
	gi 2911462	(AF046923)	serine/threonine protein kinase ...	+2	355	1.6e-58	3
	gi 458284	(U05811)	serine/threonine protein kinase [T...	+2	353	7.5e-58	3
5	gi 1000069	(U15210)	rac-alpha serine/threonine kinase ...	+2	267	2.1e-52	3
	gi 558100	(L07032)	protein kinase C-theta [Homo sapiens]	+2	274	3.1e-43	2
	gi 558099	(L01087)	protein kinase C-theta [Homo sapiens]	+2	274	3.1e-43	2
	gi 220574	(D11091)	'Protein Kinase' [Mus musculus]	+2	271	1.1e-42	2
	gi 1834511	(Y10032)	serine/threonine protein kinase [H...	+2	270	1.3e-42	2
10	gi 2463201	(AJ000512)	serine/threonine protein kinase ...	+2	270	1.3e-42	2
	gi 1673493	(Z81140)	W10G6.2 [Caenorhabditis elegans]	+2	263	1.6e-42	2
	gi 294637	(L01624)	serine/threonine protein kinase [R...	+2	269	1.7e-42	2
	gi 1136302	(D38108)	protein kinase [Schizosaccharomyce...	+2	144	3.2e-42	3
	gi 206191	(M15522)	protein kinase [Rattus norvegicus]	+2	237	1.5e-40	2
15	gi 56920	(X04139)	protein kinase C C-terminal region...	+2	237	1.5e-40	2
	gi 2996092	(U63742)	rac serine-threonine kinase homolo...	+2	189	1.9e-40	3
	gi 210068	(M80675)	gag:akt fusion protein [AKT8 retro...	+2	222	2.5e-39	4
	gi 3116064	(AJ223715)	s-sgk1 [Squalus acanthias]	+2	264	1.1e-38	2
	gi 3116066	(AJ223716)	s-sgk2 [Squalus acanthias]	+2	264	2.8e-38	2
20	gi 942578	(U22445)	serine/threonine kinase [Mus muscu...	+2	225	3.4e-38	3
	gi 2586064	(AF027183)	protein kinase C-related kinase ...	+2	244	3.8e-38	2
	gi 2652950	(Z50028)	F46F6.2 [Caenorhabditis elegans]	+2	256	4.3e-38	3
	gi 1667370	(Y07611)	protein kinase [Mus musculus]	+2	250	6.0e-38	2
	gi 1220289	(Z70043)	unknown [Schizosaccharomyces pombe]	+2	135	6.4e-38	3
25	gi 206181	(M18330)	protein kinase C delta subspecies ...	+2	235	7.1e-38	2
	gi 167718	(M59744)	protein kinase 2 [Dictyostelium di...	+2	232	8.7e-38	3
	gi 1778590	(U82935)	protein kinase C2 A isoform [Caeno...	+2	247	1.0e-37	2
	gi 1000125	(U33052)	PRK2 [Homo sapiens]	+2	245	1.1e-37	2
	gi 914100	(S75548)	protein kinase PRK2 (human, DX3 B-...	+2	245	1.1e-37	2
30	gi 200642	(M94335)	protein kinase [Mus musculus]	+2	224	1.2e-37	3
	gi 2062375	(U75358)	myeloma protein kinase [Rattus nor...	+2	245	1.5e-37	2
	gi 189510	(M60725)	p70 ribosomal S6 kinase alpha-II [...]	+2	242	1.7e-37	2
	gi 206840	(M57428)	S6 kinase [Rattus norvegicus]	+2	242	1.7e-37	2
	gi 1562	(X54415)	G3 serine/threonine kinase [Orycto...	+2	242	2.0e-37	2
35	gi 189508	(M60724)	p70 ribosomal S6 kinase alpha-I [H...	+2	242	2.0e-37	2
	gi 287807	(X65687)	serine/threonine protein kinase [M...	+2	222	2.3e-37	3
	gi 485403	(D30040)	RAC protein kinase alpha [Rattus n...	+2	222	2.3e-37	3
	gi 35481	(X61037)	human protein kinase B [Homo sapiens]	+2	222	2.8e-37	3
	gi 520587	(D10495)	protein kinase C delta-type [Homo ...]	+2	238	4.1e-37	2
40	gi 3114960	(Y13104)	Protein kinase C-related kinase (P...	+2	236	4.5e-37	2
	gi 200381	(M69042)	[Mouse protein kinase C delta mRNA...	+2	235	4.9e-37	2
	gi 189985	(L07861)	protein kinase C-delta 13 [Homo sa...	+2	238	4.9e-37	2
	gi 189680	(L07860)	protein kinase C-delta 13 [Homo sa...	+2	238	4.9e-37	2
	gi 173427	(L07637)	protein kinase C [Schizosaccharomy...	+2	252	5.4e-37	2
45	gi 303941	(D14338)	protein kinase [Schizosaccharomyce...	+2	252	5.4e-37	2
	gi 190828	(M63167)	rac protein kinase-alpha [Homo sap...	+2	222	6.1e-37	3
	gi 485405	(D30041)	RAC protein kinase beta [Rattus no...	+2	215	8.4e-37	3
	gi 414286	(U02967)	protein kinase C [Lytechinus pictus]	+2	246	8.7e-37	2
	gi 53437	(X60304)	protein kinase [Mus musculus]	+2	233	9.2e-37	2
50	gi 206169	(K03486)	protein kinase C type III [Rattus ...]	+2	237	1.2e-36	2
	gi 825510	(D26180)	PKN [Rattus norvegicus]	+2	246	1.7e-36	2
	gi 1778592	(U82936)	protein kinase C2 B isoform [Caeno...	+2	247	1.8e-36	2
	gi 1213263	(Z69795)	unknown [Schizosaccharomyces pombe]	+2	244	2.2e-36	2
	gi 631	(X61036)	bovine protein kinase B [Bos taurus]	+2	222	2.2e-36	3
55	gi 303939	(D14337)	protein kinase [Schizosaccharomyce...	+2	244	2.5e-36	2
	gi 868172	(U29376)	similar to protein kinase C [Caeno...	+2	247	2.8e-36	2
	gi 158129	(J04848)	protein kinase C [Drosophila melan...	+2	238	2.9e-36	2
	gi 1041183	(D43890)	PKN [Xenopus sp.]	+2	248	3.0e-36	2
	gi 206842	(M58340)	S6 protein kinase [Rattus norvegicus]	+2	242	3.5e-36	2
60	gi 8353	(X05076)	protein kinase C (AA 1-639) [Droso...	+2	247	4.0e-36	2
	gi 2822146	(AC002299)	Protein kinase C beta (5' partia...	+2	237	4.1e-36	2
	gi 2065190	(Y12237)	protein kinase C [Hydra vulgaris]	+2	242	4.3e-36	2

5	gi 163530	(M13973)	protein kinase C [Bos taurus]	+2	242	4.5e-36	2
	gi 1673	(X04796)	PKC-gamma (AA 1-672) [Oryctolagus ...	+2	242	4.5e-36	2
	gi 2297395	(A41791)	unnamed protein product [unidentif...	+2	242	4.5e-36	2
	gi 35483	(X52479)	protein kinase C alpha (AA 1-672) ...	+2	242	4.5e-36	2
	gi 200363	(M25811)	protein kinase C-alpha [Mus musculus]	+2	242	4.5e-36	2
10	gi 49939	(X52685)	protein kinase C [Mus musculus]	+2	242	4.5e-36	2
	gi 56914	(X07286)	protein kinase C alpha (AA 1-672) ...	+2	242	4.5e-36	2
	gi 55132	(X52684)	protein kinase C [Mus musculus]	+2	242	4.5e-36	2
	gi 3114956	(Y13099)	Serine/Threonine protein kinase [S...	+2	248	4.5e-36	2
	gi 1487920	(Z75953)	F57F5.5 [Caenorhabditis elegans]	+2	237	5.3e-36	2
15	gi 1000127	(U33053)	PRK1 [Homo sapiens]	+2	250	6.1e-36	2
	gi 914098	(S75546)	protein kinase PRK1 [human, fetal ...	+2	250	6.1e-36	2
	gi 825505	(D26181)	PKN [Homo sapiens]	+2	250	6.1e-36	2
	gi 1671	(X04795)	PKC-beta (AA 1-671) [Oryctolagus c...	+2	237	8.5e-36	2
	gi 35489	(X06318)	PKC beta 1 (AA 1-671) [Homo sapiens]	+2	237	8.5e-36	2
20	gi 206175	(M19007)	protein kinase C beta-1 [Rattus no...	+2	237	8.5e-36	2
	gi 56959	(X04439)	protein kinase C (AA 1-671) [Rattu...	+2	237	8.5e-36	2

Genbank search results using 396076E03.s2.seq (search 3 and search 4)

## Blast Results

					Reading Frame	High Score	Smallest Sum Probability P(N)	N
30	Sequences producing High-scoring Segment Pairs:							
	gi 663254	(Z48149)	probable protein kinase [Saccharom...	-3	417	8.2e-102	2	
	gi 1419952	(Z74842)	ORF YOL100w [Saccharomyces cerevis...	-3	417	8.2e-102	2	
	gi 927745	(U33050)	Ydr490cp; CAI: 0.11 [Saccharomyces...	-3	409	2.1e-99	2	
35	gi 1431588	(X99280)	protein kinase [Schizosaccharomyce...	-3	409	2.0e-92	2	
	gi 2832892	(AL021730)	hypothetical protein kinase phos...	-3	405	9.9e-81	2	
	gi 2407613	(AF017995)	3-phosphoinositide dependent pro...	-3	280	1.1e-79	3	
	gi 2505936	(Y15056)	PkB kinase [Homo sapiens]	-3	280	1.1e-79	3	
	gi 2665356	(Y15748)	PkB kinase [Rattus norvegicus]	-3	278	2.3e-79	3	
40	gi 927730	(U33050)	Ydr466wp; CAI: 0.11 [Saccharomyces...	-3	277	5.3e-74	4	
	gi 2980851	(Y07908)	serine/threonine protein kinase [D...	-3	307	4.7e-63	2	
	gi 2435556	(AF026207)	Similar to protein kinase [Caeno...	-3	259	5.6e-49	2	
	gi 1000069	(U15210)	rac-alpha serine/threonine kinase ...	-3	197	3.9e-48	3	
	gi 3043598	(AB011109)	KIAA0537 protein [Homo sapiens]	-3	233	4.4e-46	5	
45	gi 1401040	(D49836)	RAC-PK gamma [Rattus norvegicus]	-3	198	2.2e-45	4	
	gi 35479	(X07767)	protein kinase catalytic subunit t...	-3	188	6.2e-45	3	
	gi 191175	(M63311)	cAMP-dependent protein kinase alph...	-3	188	6.2e-45	3	
	gi 200367	(M12303)	cAMP-dependent protein kinase cata...	-3	190	1.2e-44	3	
	gi 387513	(M19960)	cAMP-dependent protein kinase alph...	-3	190	1.2e-44	3	
50	gi 1890142	(D83380)	catalytic subunit of cAMP-dependen...	-3	185	1.3e-44	3	
	gi 2253148	(Z82096)	ZK909.2a [Caenorhabditis elegans]	-3	185	1.9e-44	3	
	gi 2299249	(A44422)	unnamed protein product [Eimeria m...	-3	148	2.3e-44	5	
	gi 56912	(X57986)	cAMP-dependent protein kinase subu...	-3	188	2.4e-44	3	
	gi 288120	(X53261)	cAMP-dependent protein kinase cata...	-3	184	2.5e-44	3	
55	gi 2253149	(Z82096)	ZK909.2b [Caenorhabditis elegans]	-3	185	3.4e-44	3	
	gi 633	(X67154)	protein kinase [Bos taurus]	-3	188	4.8e-44	3	
	gi 2760821	(L06196)	serine/threonine protein kinase [E...	-3	200	5.3e-44	3	
	gi 1673493	(Z81140)	W10G6.2 [Caenorhabditis elegans]	-3	269	1.1e-43	2	
	gi 1911	(X05998)	C-beta subunit (338 AA) [Sus scrofa]	-3	187	1.4e-43	3	
60	gi 2632252	(Y12464)	serine/threonine kinase [Sorghum b...	-3	185	1.6e-43	4	
	gi 773642	(U23730)	vinclozolin resistance protein [Us...	-3	191	2.6e-43	4	

	gi 200642	(M94335)	protein kinase [Mus musculus]	-3	132	2.8e-43	5
	gi 2213667	(U83459)	Rim15p [Saccharomyces cerevisiae]	-3	248	2.8e-43	4
	gi 836721	(D50617)	YFL033C [Saccharomyces cerevisiae]	-3	248	2.8e-43	4
5	gi 603542	(X83510)	RAC protein kinase DRAC-PK66 [Dros...	-3	187	3.7e-43	4
	gi 398924	(Z26242)	Dakt1 serine-threonine protein kin...	-3	187	3.7e-43	4
	gi 166600	(M93023)	SNF1-related protein kinase [Arabi...	-3	193	4.0e-43	4
	gi 1742969	(X94757)	ser/thr protein kinase [Arabidopsi...	-3	193	4.0e-43	4
	gi 1279425	(Z71757)	calmodulin-domain protein kinase [...	-3	145	4.4e-43	5
10	gi 178326	(M95936)	protein serine/threonine kinase [H...	-3	191	6.5e-43	4
	gi 903942	(U29608)	LATS [Drosophila melanogaster]	-3	210	7.3e-43	4
	gi 755008	(L39837)	tumor suppressor [Drosophila melan...	-3	210	7.3e-43	4
	gi 1335643	(X83510)	RAC protein kinase DRAC-PK85 [Dros...	-3	187	9.3e-43	4
	gi 337491	(M77198)	rac protein kinase-beta [Homo sapi...	-3	191	1.2e-42	4
15	gi 287807	(X65687)	serine/threonine protein kinase [M...	-3	127	1.4e-42	5
	gi 485403	(D30040)	RAC protein kinase alpha [Rattus n...	-3	127	1.4e-42	5
	gi 496385	(D26602)	protein kinase [Nicotiana tabacum]	-3	189	1.4e-42	4
	gi 1743009	(Y10036)	SNF1-related protein kinase [Cucum...	-3	192	1.8e-42	4
	gi 1935916	(U83797)	StubSNF1 protein [Solanum tuberosum]	-3	196	7.0e-42	4
20	gi 191177	(M63312)	cAMP-dependent protein kinase beta...	-3	184	1.8e-41	3
	gi 200387	(J02626)	cAMP-dependent protein kinase beta...	-3	184	1.8e-41	3
	gi 220704	(D10770)	cAMP-dependent protein kinase cata...	-3	184	1.8e-41	3
	gi 163532	(J02647)	protein kinase beta-catalytic subu...	-3	183	1.8e-41	3
	gi 189983	(M34181)	cAMP-dependent protein kinase cata...	-3	183	1.8e-41	3
25	gi 942578	(U22445)	serine/threonine kinase [Mus muscu...	-3	188	3.0e-41	4
	gi 210068	(M80675)	gag:akt fusion protein [AKT8 retro...	-3	127	5.5e-41	5
	gi 172175	(J02665)	protein kinase [Saccharomyces cere...	-3	186	5.5e-41	3
	gi 173009	(M17072)	cAMP-dependent protein kinase subu...	-3	186	5.5e-41	3
	gi 1008352	(Z49439)	ORF YJL164c [Saccharomyces cerevis...	-3	186	5.5e-41	3
30	gi 162787	(M60482)	cAMP-dependent protein kinase II-b...	-3	183	5.5e-41	3
	gi 407516	(Z26878)	unknown [Saccharomyces cerevisiae]	-3	187	5.6e-41	3
	gi 486291	(Z28166)	ORF YKL166c [Saccharomyces cerevis...	-3	187	5.6e-41	3
	gi 156233	(M37120)	cAMP-dependent protein kinase cata...	-3	178	7.4e-41	3
	gi 173011	(M17073)	cAMP-dependent protein kinase subu...	-3	188	8.3e-41	
	3..						